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A/RE
REISSUE LITIGATION

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PTO/SB/50 (4/98)
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Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

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BROADENING REISSUE PATENT APPLICATION TRANSMITTAL

Address to:

**Assistant Commissioner for Patents
Box Patent Application
Washington, DC 20231**

Attorney Docket No.	MONY:140
First Named Inventor	Gerard F. Barry
Original Patent Number	5,776,760
Original Patent Issue Date (Month/Day/Year)	July 7, 1998
Express Mail Label No.	EL521270395US

APPLICATION FOR REISSUE OF:
(check applicable box)

☒ Utility Patent

☐ Design Patent

☐ Plant Patent

APPLICATION ELEMENTS

- ☒ * Fee Transmittal Form (PTO/SB/56)
(Submit an original, and a duplicate for fee processing)
- ☒ Specification and Claims (amended, if appropriate)
including broadened reissue claims
- ☒ Drawing(s) (proposed amendments, if appropriate)
- ☐ Reissue Oath / Declaration (original or copy)
(37 C.F.R. § 1.175)(PTO/SB/51 or 52)
- Original U.S. Patent
☐ Offer to Surrender Original Patent (37 C.F.R. § 1.178)
(PTO/SB/53 OR PTO/SB/54)
or
☐ Ribboned Original Patent Grant
☐ Affidavit / Declaration of Loss (PTO/SB/55)
- Original U.S. Patent currently assigned?
☒ Yes ☐ No
(If Yes, check applicable box(es))
☐ Written Consent of all Assignees (PTO/SB/53 or 54)
☐ 37 C.F.R. § 3.73(b) Statement ☐ Power of Attorney

ACCOMPANYING APPLICATION PARTS

- ☐ Foreign Priority Claim (35 U.S.C. 119)
(if applicable)
- ☐ Information Disclosure Statement (IDS)/PTO-1449 ☐ Copies of IDS Citations
- ☐ English Translation of Reissue Oath/Declaration
(if applicable)
- ☐ * Small Entity Statement(s) ☐ Statement filed in prior application, Status still proper and desired (PTO/SB/09-12)
- ☐ Preliminary Amendment
- ☒ Return Receipt Postcard (MPEP 503)
(Should be specifically itemized)
- ☐ Other: _____

*** NOTE FOR ITEMS 1 & 10: IN ORDER TO BE ENTITLED TO PAY SMALL ENTITY FEES, A SMALL ENTITY STATEMENT IS REQUIRED (37 C.F.R. § 1.27), EXCEPT IF ONE FILED IN A PRIOR APPLICATION IS RELIED UPON (37 C.F.R. § 1.28).**


14. CORRESPONDENCE ADDRESS

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Name	Janelle D. Waack				
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NAME (Print/Type)	Janelle D. Waack	Registration No. (Attorney/Agent)	36,300
Signature		Date	July 7, 2000

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REISSUE APPLICATION FEE TRANSMITTAL FORM	Docket Number (Optional) <div style="text-align: center; font-size: 1.2em;">MONY:140</div>
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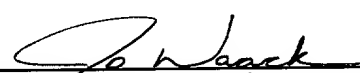
Claims as Filed - Part 1								
Claims in Patent	For	Number Filed in Reissue Application	(3) Number Extra	Small Entity		Other than a Small Entity		
				Rate	Fee	Rate	Fee	
(A) 2	Total Claims (37 CFR 1.16(j))	(B) 20	**** 18 =	x \$	=	or	x \$ 18 =	\$324.00
(C) 1	Independent Claims (37 CFR 1.16(i))	(D) 13	* = 12	x \$	=		x \$ 78 =	\$936.00
Basic Fee (37 CFR 1.16(h))					\$		\$ 690.00	
Total Filing Fee					\$		OR \$ 1,950.00	

Claims as Amended - Part 2								
	(1) Claims Remaining After Amendment		(2) Highest Number Previously Paid For	(3) Extra Claims Present	Small Entity		Other than a Small Entity	
					Rate	Fee	Rate	Fee
Total Claims (37 CFR 1.16(j))	***	MINUS	**	* = 0	x \$	=	or	x \$ = 0
Independent Claims (37 CFR 1.16(i))	***	MINUS	*****	= 0	x \$	=		x \$ = 0
Total Additional Fee					\$		OR \$ 0	

* If the entry in (D) is less than the entry in (C), Write "0" in column 3.
 ** If the "Highest Number of Total Claims Previously Paid For" is less than 20, Write "20" in this space.
 *** After any cancellation of claims
 **** If "A" is greater than 20, use (B - A); if "A" is 20 or less, use (B - 20).
 ***** "Highest Number of Independent Claims Previously Paid For" or Number of Independent Claims in Patent (C).

- ☒ Please charge Deposit Account No. 01-2508/MONY:140/WAA in the amount of \$1,950.00.
 A duplicate copy of this sheet is enclosed.
- ☒ The Commissioner is hereby authorized to charge any additional fees under 37 CFR 1.16 or 1.17 which may be required, or credit any overpayment to Deposit Account No. 01-2508/MONY:140/WAA.
 A duplicate copy of this sheet is enclosed.
- ☐ A check in the amount of \$ _____ to cover the filing / additional fee is enclosed.

July 7, 2000
 Date


 Signature of Applicant, Attorney or Agent of Record

Janelle D. Waack, Reg. No. 36,300
 Typed or Printed Name

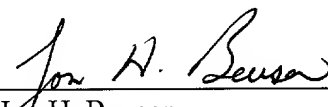


Atty Docket: MONY:140/WAA
11914.0140.NPUS00
38-21(13560)B US

assignments is attached. Also enclosed is certification for the recent name change of MONSANTO COMPANY to PHARMACIA CORPORATION. These documents evidence the chain of title from the inventors to PHARMACIA CORPORATION.

Further, PHARMACIA CORPORATION, as assignee of U.S. Patent No. 5,776,760, consents to the filing of the present application for the reissue of U.S. Patent No. 5,776,760.

I am authorized to act on behalf of assignee PHARMACIA CORPORATION.



Jon H. Beusen

Registration No. 30,610

PHARMACIA CORPORATION

Date: Sept. 5, 2000

Jon H. Beusen
Intellectual Property Counsel
Authorized to sign this document for
Pharmacia Corporation (formerly Monsanto Company)
by resolution dated February 25, 2000
of the Board of Directors



SECRET

CERTIFICATE OF MAILING 37 C.F.R. 1.8	
I hereby certify that this correspondence is being deposited with the U.S. Postal Service with sufficient postage as First Class Mail in an envelope addressed to: BOX MISSING PARTS, Assistant Commissioner for Patents, Washington, D.C. 20231, on the date below:	
9.19.00 Date	 Signature

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Gerard F. Barry
Ganesh M. Kishore

Serial No.: 09/612,404

Filed: July 7, 2000

For: GLYPHOSATE TOLERANT PLANTS

Examiner: not yet assigned

Atty. Dkt. No.: MONY: 140/WAA
11914.0140.NPUS00
38-21(13560)B US

RESPONSE TO NOTICE TO FILE MISSING PARTS

BOX MISSING PARTS

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

In response to the Notice to File Missing Parts of Application, dated August 3, 2000, enclosed are:

- (a) Reissue Application Declaration executed by inventors Gerard F. Barry and Ganesh M. Kishore;
- (b) Certification under 37 C.F.R. § 3.73 and Consent of Assignee to Reissue Application; and
- (c) A copy of the Notice to File Missing Parts of Application-Filing Date Granted, dated August 3, 2000.

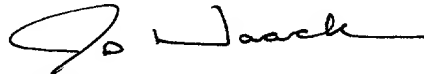
Also enclosed is:

(d) Reissue Application by the Assignee, Offer to Surrender Patent.

Please charge the surcharge for late filing (\$130.00) and any additional required fee to Deposit Account No. 01-2508/MONY:140/WAA.

Please date stamp and return the accompanying postcard to evidence receipt of these documents.

Respectfully submitted,



Janelle D. Waack
Reg. No. 36,300

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Attorney for Assignee
PHARMACIA CORPORATION

Date: September 19, 2000



FORMALITIES LETTER



OC000000005294825



UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office

Address COMMISSIONER OF PATENT AND TRADEMARKS
Washington, D C 20231

APPLICATION NUMBER	FILING/RECEIPT DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NUMBER
09/612,404	07/07/2000	Gerard F. Barry	MONY:140

Janelle D Waack
Howrey Simon Arnold & White LLP
750 Bering Drive
Houston, TX 77057-2198

Date Mailed: 08/03/2000

NOTICE TO FILE MISSING PARTS OF REISSUE APPLICATION

Filing Date Granted

An application number and filing date have been accorded to this reissue application. The item(s) indicated below, however, are missing. Applicant is given TWO MONTHS from the date of this Notice within which to file all required items and pay any fees required below to avoid abandonment. Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136(a).

- The oath or declaration is missing.
A properly signed oath or declaration in compliance with 37 CFR 1.63, identifying the application by the above Application Number and Filing Date, is required.
- To avoid abandonment, a late filing fee or oath or declaration surcharge as set forth in 37 CFR 1.16(e) of \$130 for a non-small entity, must be submitted with the missing items identified in this letter.
- Assignee's statement under 37 CFR 3.73(b) establishing ownership of the patent is missing. 37 CFR 1.172 requires that all assignees consenting to the reissue application establish their ownership interest in the patent by filing in the reissue application a statement in accordance with 37 CFR 3.73(b).
- **The balance due by applicant is \$ 130.**

*A copy of this notice **MUST** be returned with the reply.*

K. Nelson

Customer Service Center

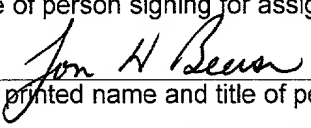
Initial Patent Examination Division (703) 308-1202

PART 2 - COPY TO BE RETURNED WITH RESPONSE

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REISSUE APPLICATION BY THE ASSIGNEE, OFFER TO SURRENDER PATENT		Docket Number (Optional) MONY:140
This is part of the application for a reissue patent based on the original patent identified below.		
Name of Patentee(s): Gerard F. Barry and Ganesh M. Kishore		
Patent Number U.S. Patent No. 5,776,760	Date Patent Issued July 7, 1998	
Title of Invention Glyphosate Tolerant Plants		
<u>Pharmacia Corporation</u> is the assignee of the entire interest in the original patent. I offer to surrender the original patent. <input checked="" type="checkbox"/> A certificate under 37 CFR 3.73(b) is attached. I am authorized to act on behalf of the assignee.		
I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application, any patent issued thereon, or any patent to which this declaration is directed.		
Name of assignee Pharmacia Corporation		
Signature of person signing for assignee 	Date Sept 5, 2000	
Typed or printed name and title of person signing for assignee Jon H. Beusen		
Jon H. Beusen Intellectual Property Counsel Authorized to sign this document for Pharmacia Corporation (formerly Monsanto Company) by resolution dated February 25, 2000 of the Board of Directors		

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GLYPHOSATE TOLERANT PLANTS

This is a continuation of application Ser. No. 08/391,339, filed Feb. 21, 1995; which is a continuation of application Ser. No. 08/156,968, filed Nov. 23, 1993, now abandoned, which is a continuation of application Ser. No. 07/717,370, filed Jun. 24, 1991, now abandoned, which is a continuation in part of application Ser. No. 07/543,236, filed Jun. 25, 1990, now abandoned.

BACKGROUND OF THE INVENTION

Recent advances in genetic engineering have provided the requisite tools to transform plants to contain foreign genes. It is now possible to produce plants which have unique characteristics of agronomic importance. Certainly, one such advantageous trait is more cost effective, environmentally compatible weed control via herbicide tolerance. Herbicide-tolerant plants may reduce the need for tillage to control weeds thereby effectively reducing soil erosion.

One herbicide which is the subject of much investigation in this regard is N-phosphonomethyl-glycine commonly referred to as glyphosate. Glyphosate inhibits the shikimic acid pathway which leads to the biosynthesis of aromatic compounds including amino acids and vitamins. Specifically, glyphosate inhibits the conversion of phosphoenolpyruvic acid and 3-phosphoshikimic acid to 5-enolpyruvyl-3-phosphoshikimic acid by inhibiting the enzyme 5-enolpyruvyl-3-phosphoshikimic acid synthase (EPSP synthase or EPSPS).

It has been shown that glyphosate tolerant plants can be produced by inserting into the genome of the plant the capacity to produce a higher level of EPSP synthase which enzyme is preferably glyphosate tolerant (Shah et al., 1986). The introduction into plants of glyphosate degradation gene (s) could provide a means of conferring glyphosate tolerance to plants and/or to augment the tolerance of transgenic plants already expressing a glyphosate tolerant EPSP synthase depending upon the physiological effects of the degradation products.

Glyphosate metabolism (degradation) has been examined in a wide variety of plants and little degradation has been reported in most of those studies. In those instances where degradation has been reported, the initial breakdown product is usually aminomethylphosphonate (AMPA) (Coupland, 1985; Marshall et al., 1987). In these instances, it is not clear if glyphosate is metabolized by the plant or the contaminating microbes on the leaf surface to which glyphosate was applied. AMPA has been reported to be much less phytotoxic than glyphosate for most plant species (Franz, 1985) but not for all plant species (Maier, 1983; Tanaka et al., 1988). Glyphosate degradation in soils is much more extensive and rapid (Torstensson, 1985). The principal breakdown product identified is AMPA (Rueppel et al., 1977; Nomura and Hilton, 1977); a phosphonate that can be metabolized by a wide variety of microorganisms (Zeleznick et al., 1963; Mastalerz et al., 1965; Cook et al., 1978; Daughton et al., 1979a; 1979b; 1979c; Wackett et al., 1987a). A number of pure cultures of bacteria have been identified that degrade glyphosate by one of the two known routes (Moore et al., 1983; Talbot et al., 1984; Shinabarger and Braymer, 1986; Balthazor and Hallas, 1986; Kishore and Jacob, 1987; Wackett et al., 1987a; Pipke et al., 1987a; Pipke et al., 1987b; Hallas et al., 1988; Jacob et al., 1985 and 1988; Pipke and Amrhein, 1988; Quinn et al., 1988 and 1989; Lerbs et al., 1990; Schowanek and Verstraete, 1990; Weidhase et al., 1990; Liu et al., 1991). A route involving a "C-P lyase" that

degrades glyphosate to sarcosine and inorganic orthophosphate (Pi) has been reported for a *Pseudomonas* sp. (Shinabarger and Braymer, 1986; Kishore and Jacob, 1987) and an *Arthrobacter* sp. (Pipke et al., 1987b). Pure cultures capable of degrading glyphosate to AMPA have been reported for a *Flavobacterium* sp. (Balthazor and Hallas, 1986), for a *Pseudomonas* sp. (Jacob et al., 1988) and for *Arthrobacter atrocyaneus* (Pipke and Amrhein, 1988). In addition, a large number of isolates that convert glyphosate to AMPA have been identified from industrial activated sludges that treat glyphosate wastes (Hallas et al., 1988). However, the number and nature of bacterial genes responsible for these degradations have not been heretofore determined nor have the gene(s) been isolated.

Hence, in one aspect, an object of the present invention is to provide novel genes which encode a glyphosate metabolizing enzyme which converts glyphosate to aminomethylphosphonate and glyoxylate.

Another object is to enhance the activity of the glyphosate metabolizing enzyme against glyphosate by replacement of specific amino acid residues.

Another object of the present invention is to provide genetically modified plants which express a gene which encodes a glyphosate metabolizing enzyme and which exhibit enhanced tolerance to glyphosate herbicide.

Another object is to demonstrate that a glyphosate metabolizing enzyme can be targeted to plastids using chloroplast transit peptides and the plastid targeted enzyme confers high level glyphosate tolerance.

A further object is to provide a method for selecting transformed plant tissue using the glyphosate metabolizing enzyme as the selectable marker in the presence of inhibitory concentrations of glyphosate.

These and other objects, aspects and features of the present invention will become evident to those skilled in the art from the following description and working examples.

SUMMARY OF THE INVENTION

The present invention provides structural DNA constructs which encode a glyphosate oxido-reductase enzyme and which are useful in producing glyphosate degradation capability in heterologous microorganisms (e.g. bacteria and plants) and in producing glyphosate tolerant plants.

In accomplishing the foregoing, there is provided, in accordance with one aspect of the present invention, a method of producing genetically transformed plants which are tolerant toward glyphosate herbicide, comprising the steps of:

- (a) inserting into the genome of a plant cell a recombinant, double-stranded DNA molecule comprising
 - (i) a promoter which functions in plant cells to cause the production of an RNA sequence,
 - (ii) a structural DNA sequence that causes the production of an RNA sequence which encodes a glyphosate oxidoreductase enzyme,
 - (iii) a 3' non-translated DNA sequence which functions in plant cells to cause the addition of polyadenylated nucleotides to the 3' end of the RNA sequence;
 where the promoter is heterologous with respect to the coding sequence and adapted to cause sufficient expression of said enzyme in plant tissue, including meristematic tissue, to enhance the glyphosate resistance of a plant cell transformed with said gene;
- (b) obtaining a transformed plant cell; and

3

(c) regenerating from the transformed plant cell a genetically transformed plant which has increased tolerance to glyphosate herbicide.

In accordance with another aspect of the present invention, there is provided a recombinant, double-stranded DNA molecule comprising in sequence:

- (a) a promoter which functions in plants to cause the production of an RNA sequence;
- (b) a structural DNA sequence that causes the production of an RNA sequence which encodes a glyphosate oxidoreductase enzyme; and
- (c) a 3' non-translated region which functions in plants to cause the addition of polyadenylated nucleotides to the 3' end of the RNA sequence. There has also been provided, in accordance with another aspect of the present invention, bacterial and transformed plant cells that contain, respectively, DNA comprised of the above-mentioned elements (a), (b) and (c).

In accordance with yet another aspect of the present invention, differentiated plants have been provided that comprise transformed plant cells, as described above, which exhibit tolerance toward glyphosate herbicide.

In accordance with still another aspect of the present invention, there has been provided a method for selectively controlling weeds in a field containing a crop having planted crop seeds or plants comprising the steps of:

- (a) planting said crop seeds or plants which are glyphosate tolerant as a result of a recombinant double-stranded DNA molecule being inserted into said crop seed or plant, said DNA molecule having
 - (i) a promoter sequence which functions in plants to cause the production of an RNA sequence.
 - (ii) a structural DNA sequence which causes the production of RNA which encodes a glyphosate oxidoreductase enzyme.
 - (iii) a 3' non-translated region which encodes a polyadenylation signal which functions in plants to cause the addition of polyadenylated nucleotides to the 3' end of the RNA sequence.

where the promoter is heterologous with respect to the coding sequence and adapted to cause sufficient expression of said enzyme in plant tissue, including meristematic tissue, to enhance the glyphosate tolerance of a plant cell transformed with said gene; and

- (b) applying to said crop and weeds in said field a sufficient amount of glyphosate herbicide to control said weeds without significantly affecting said crop.

In a particularly preferred embodiment the double-stranded DNA molecule comprising a gene for plant expression comprises a structural DNA sequence encoding a fusion polypeptide containing an amino-terminal chloroplast transit peptide which is capable of causing importation of the carboxy-terminal glyphosate oxidoreductase enzyme into the chloroplast of the plant cell expressing said gene.

A further embodiment of the present invention is the use of the glyphosate oxidoreductase gene as a selectable marker to select and identify transformed plant tissue.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A-1B show the DNA sequence for the full-length promoter of figwort mosaic virus (FMV), (SEQ ID NO:1), including an exemplary 5' non-translated leader sequence (SEQ ID NO:2).

FIGS. 2A-2G show the structural DNA sequence for a glyphosate oxidoreductase gene from bacterial isolate LBAA SEQ ID NO:3.

such as the cauliflower mosaic virus (CaMV) 19S and 35S promoters and the figwort mosaic virus (FMV) 35S promoter, the light-inducible promoter from the small sub-unit of ribulose bis-phosphate carboxylase (ssRUBISCO, a very abundant plant polypeptide). All of these promoters have been used to create various types of DNA constructs which have been expressed in plants; see, e.g., PCT publication WO 84/02913 (Rogers et al., Monsanto).

Promoters which are known or are found to cause transcription of DNA in plant cells can be used in the present invention. Such promoters may be obtained from a variety of sources such as plants and plant DNA viruses and include, but are not limited to, the CaMV35S and FMV35S promoters and promoters isolated from plant genes such as ssRUBISCO genes or the chlorophyll a/b binding proteins. As described below, it is preferred that the particular promoter selected should be capable of causing sufficient expression to result in the production of an effective amount of glyphosate oxidoreductase to render the plant substantially tolerant to glyphosate herbicides. The amount of glyphosate oxidoreductase needed to induce the desired tolerance may vary with the plant species.

It is preferred that the promoters utilized have relatively high expression in all meristematic tissues in addition to other tissues inasmuch as it is now known that glyphosate is translocated and accumulated in this type of plant tissue. Alternatively, a combination of chimeric genes can be used to cumulatively result in the necessary overall expression level of glyphosate oxidoreductase enzyme to result in the glyphosate tolerant phenotype.

The MRNA produced by a DNA construct of the present invention also contains a 5' non-translated leader sequence. This sequence can be derived from the promoter selected to express the gene, and can be specifically modified so as to increase translation of the mRNA. The 5' non-translated regions can also be obtained from viral RNAs, from suitable eukaryotic genes, or from a synthetic gene sequence. The present invention is not limited to constructs, as presented in the following examples, wherein the non-translated region is derived from both the 5' non-translated sequence that accompanies the promoter sequence and part of the 5' non-translated region of the virus coat protein gene. Rather, the non-translated leader sequence can be derived from an unrelated promoter or coding sequence as discussed above.

A preferred promoter for use in the present invention is the full-length transcript (35S) promoter from the figwort mosaic virus (FMV) which functions as a strong and uniform promoter for chimeric genes inserted into plants, particularly dicotyledons. In general, the resulting transgenic plants express the protein encoded by the inserted gene at a higher and more uniform level throughout the tissues and cells than the same gene driven by an enhanced CaMV35S promoter. Referring to FIG. 1, the DNA sequence of the promoter is located between nucleotides 6368 and 6930 (SEQ ID NO:1) of the FMV genome. A 5' non-translated leader sequence is preferably coupled with the promoter and an exemplary leader sequence (SEQ ID NO:2) is shown in FIG. 1. The leader sequence can be from the FMV genome itself or can be from a source other than FMV.

The 3' non-translated region of the chimeric plant gene contains a polyadenylation signal which functions in plants to cause the addition of polyadenylate nucleotides to the 3' end of the RNA. Examples of suitable 3' regions are (1) the 3' transcribed, non-translated regions containing the polyadenylation signal of *Agrobacterium* tumor-inducing (Ti) plasmid genes, such as the nopaline synthase (NOS) gene.

and (2) plant genes such as the soybean storage protein genes and the small subunit of the ribulose-1,5-bisphosphate carboxylase (ssRUBISCO) gene. An example of a preferred 3' region is that from the ssRUBISCO gene from pea (E9), described in greater detail in the examples below.

The DNA constructs of the present invention also contain a structural coding sequence in double-stranded DNA form, which encodes a glyphosate oxidoreductase enzyme which converts glyphosate to aminomethylphosphonate and glyoxylate.

Summary of the Glyphosate Oxidoreductase Reaction

The enzyme glyphosate oxidoreductase catalyzes the cleavage of the C-N bond of glyphosate yielding aminomethyl phosphonate (AMPA) and glyoxylate as the reaction products. Under aerobic conditions, oxygen is utilized as a cosubstrate for the reaction. Other electron carriers such as phenazine methosulfate and ubiquinone stimulate the reaction under aerobic conditions. In the absence of oxygen, these compounds act as electron acceptors.

The enzymatic reaction can be assayed by oxygen uptake using an oxygen electrode. The glyphosate oxido-reductase from LBAA does not produce hydrogen peroxide as a product of oxygen reduction. This enzyme has a stoichiometry of two moles of glyphosate oxidized per mole of oxygen consumed and produces two moles each of AMPA and glyoxylate as reaction products.

An alternate method for the assay of glyphosate oxidoreductase involves reaction of the sample with 2,4-dinitrophenylhydrazine and determination of the amount of the glyoxylate-2,4-dinitrophenylhydrazone by HPLC analysis as described in detail in a later section.

A third method for the assay of glyphosate oxidoreductase consists of using [3-¹⁴C]-glyphosate as a substrate; the radioactive AMPA produced by the enzyme is separated from the substrate by HPLC on anion exchange column as described later. The radioactivity associated with AMPA is a measure of the extent of the glyphosate oxidoreductase reaction.

Glyphosate oxidoreductase from LBAA is a flavoprotein using FAD as a cofactor. One of the mechanisms we have proposed for the reaction catalyzed by this enzyme involves the reduction of the FAD at the active site of the enzyme by glyphosate. This leads to the formation of reduced FAD and a Schiff base of aminomethylphosphonate with glyoxylate. The Schiff base is hydrated by water and hydrolyzed to its components, AMPA and glyoxylate. The reduced flavin is reoxidized by molecular oxygen. We suggest that during the process of reoxidation of reduced FAD, an oxygenated flavin is produced as an intermediate. This flavin intermediate may catalyze the oxygenation of glyphosate yielding AMPA and glyoxylate. This hypothesis is in accordance with the observed stoichiometry and our inability to detect hydrogen peroxide in the reaction mixture.

In addition to glyphosate, glyphosate oxido-reductase from LBAA oxidizes iminodiacetic acid (IDA) to glycine and glyoxylate. The rate of the reaction with IDA is significantly faster than with glyphosate.

Isolation of Efficient Glyphosate-to-AMPA Degrading Bacterium

Bacteria capable of degrading glyphosate are known. (Hallas et al., 1988; Malik et al., 1989). A number of these bacteria were screened for the rapid degradation of glyphosate in the following manner: twenty three bacterial isolates were transferred from TSA (Trypticase Soya Agar; BBL) plates into medium A consisting of Dworkin-Foster salts medium containing glucose, gluconate and citrate (each at 0.1%) as carbon source and containing glyphosate at 0.1 mM as the phosphorous source.

Dworkin-Foster minimal medium was made up by combining in 1 liter (with autoclaved H₂O) 1 ml each of A, B and C and 10 ml of D, thiamine HCl (5 mg), C-sources to final concentrations of 0.1% each and P-source (glyphosate or other phosphonates or Pi) to the required concentration:

A. D-F Salts (1000× stock; per 100 ml; autoclaved):

H₃BO₃ 1 mg
 MnSO₄·7H₂O 1 mg
 ZnSO₄·7H₂O 12.5 mg
 CuSO₄·5H₂O 8 mg
 NaMoO₃·3H₂O 1.7 mg

B. FeSO₄·7H₂O (1000× stock; per 100 ml; autoclaved)
 0.1 g

C. MgSO₄·7H₂O (1000× stock; per 100 ml; autoclaved)
 20 g

D. (NH₄)₂SO₄ (100× stock; per 100 ml; autoclaved)
 20 g

Yeast Extract (YE; Difco) was added to a final concentration of

0.01 or 0.001%.

Each 1 ml of culture medium also contained approximately 200,000 cpm [3-¹⁴C]glyphosate (Amersham; CFA.745). The cultures were incubated with shaking at 30° C. Isolate LBAA showed significant growth at day one, while other test cultures showed little growth before day three. Determination of radioactivity (by scintillation counting) in the culture, cell pellet and culture supernatant (at day 4) revealed that total ¹⁴C radioactivity had decreased and that remaining was partitioned ~1:1 in the supernatant and pellet, indicating that significant uptake and metabolism of glyphosate had taken place.

TABLE I

Glyphosate Metabolism by LBAA Culture	
Sample	¹⁴ C cpm
control	18,631
LBAA culture	11,327
LBAA supernatant	6,007
LBAA cells	4,932

At day five, 75 µl of the culture supernatant of all test cultures was analyzed by HPLC as follows: a SYNCHROPAK™AX100 anion exchange column (P.J. Cobert) was used and the mobile phase consisted of 65 mM KH₂PO₄ (pH 5.5 with NaOH; depending on the needs of the experiment the concentration of the phosphate buffer was varied from 50 to 75 mM in order to alter the retention times of the material), run isocratically and the eluted material monitored continuously using a radioactive detector. This analysis revealed, in one isolate in particular (LBAA), that the glyphosate peak (Retention Time [RT]=7.0 minutes in this analysis) was completely absent and a new peak of radioactivity had appeared, with the same RT as methylamine or N-acetylmethylamine (RT =3.5 minutes). The collection of bacteria, of which strain LBAA formed a part, had been characterized as degrading glyphosate to AMPA (Hallas et al., 1988); the detection of methylamine or N-Acetylmethylamine suggested that the AMPA or N-AcetylAMPA was being metabolized by the LBAA "C-P lyase" activity to release the phosphate required for growth in this experiment. Strain LBAA was examined in greater detail.

Conversion of Glyphosate to AMPA in Microbial Isolates

For clarity and brevity of disclosure, the following description of the isolation of genes encoding glyphosate

oxidoreductase enzymes is directed to the isolation of such a gene from a bacterial isolate (LBAA). Those skilled in the art will recognize that the same or a similar strategy can be utilized to isolate such genes from other microbial isolates.

- 5 The glyphosate degradation pathway was characterized in resting cells of glyphosate-grown strain LBAA as follows: the cells from a 100 ml culture of LBAA, grown in DF medium with glucose, gluconate and citrate as carbon sources and with thiamine and Yeast Extract (0.01%) to supply trace requirements (=medium DF3S) and with glyphosate at 0.2 mM as a phosphorous source, were harvested at Klett=200, washed twice with 20 ml of DF3S medium and the equivalent of 20 ml cells resuspended in 100 ul of the same medium containing [3-¹⁴C] glyphosate (2.5 ul of 52 mCi/mmol). The cell mix was incubated at 30° C. with shaking and samples (20 ul) were withdrawn at intervals. The samples were centrifuged and both the supernatant and cell pellets were analyzed by HPLC (the cell pellets were resuspended in 100 ul of acid-DF3S [=DF3S, 0.65N HCl],
10 boiled for 5 minutes, centrifuged briefly and this supernatant was analyzed; an acidified glyphosate control was also examined). In two hours the amount of radioactivity in the glyphosate peak (RT=7.8 minutes) in the supernatant had decreased to ~33% of the starting level; about 3% of the
15 glyphosate was found within the cell. Material co-eluting with the methylamine standard accounted for ~5% of the starting counts in the supernatant and for ~1.5% in the cell pellet. A new peak, accounting for ~1.5% of the starting radioactivity with a RT of 7.7 minutes (glyphosate RT=8.9 minutes upon acidification in this experiment) was identified in the cell contents. The large decrease in overall radioactivity also suggested that the glyphosate was extensively metabolized in this experiment. The pathway was elucidated further in a subsequent experiment where the metabolism of
20 [¹⁴C] AMPA was compared to that of [3-¹⁴C] glyphosate (as above) in resting cells harvested at Klett 165 and resuspended at the equivalent to 15 ml cells per 100 ul DF3S medium. The samples were analyzed by HPLC and consisted of whole cultures acidified and treated as described
25 above. Within the first two hours of the glyphosate experiment, 25% of the radioactivity was found in the methylamine/N-acetylmethylamine peak (RT=4.8 minutes), 12.5% as AMPA (RT=6.4 minutes), 30% as the peak alluded to above (RT=9.4 minutes) and 30% as glyphosate (RT=11.8 minutes). In the AMPA experiment 15% of the radioactivity was found as N-acetylmethylamine/methyl-amine, 59% as AMPA and 18% in the peak with RT=9.4 minutes. The modified form of AMPA was identified as N-acetylAMPA. A similar acetylation step has been inferred from the products
30 identified in *E. coli* growing in aminomethylphosphonates as sole sources of P (Avila et al., 1987). These data indicated that the glyphosate degradation pathway in LBAA is glyphosate → AMPA (→ methylamine) → N-acetylAMPA → N-acetylmethylamine.
35 Cloning of the Glyphosate Oxidoreductase Gene(s) in *E. coli*

Having established the glyphosate-to-AMPA conversion in strain LBAA, a direct approach for the cloning of the gene(s) involved in this conversion into *E. coli* was investigated. Cloning and genetic techniques, unless otherwise indicated, were generally those described (Maniatis et al., 1982). The cloning strategy was as follows: introduction of a cosmid bank of strain LBAA into *E. coli* and selection for the glyphosate-to-AMPA gene(s) by requiring growth on
40 glyphosate as a phosphorous (P) source. This selection relied on the use of AMPA generated by the glyphosate metabolizing enzyme as a P source, following the release of the Pi

002020 "4027960

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from the AMPA by the *E. coli* "C-P lyase." Most *E. coli* strains are incapable of utilizing phosphonates as P sources upon initial challenge, however these strains usually adapt rapidly, independently of RecA, to utilize phosphonates (become Mpu+) (Wackett et al., 1987b). *E. coli* Mpu+ was isolated from *E. coli* SR200 (Leu-, Pro-recA, hsdR, supE, Smr, tonA) as follows: an aliquot of a fresh L-broth culture of *E. coli* SR200 was plated on MOPS (Neidhardt et al., 1974) complete agar (i.e., contains L-leucine and L-proline at 25 ug/ml and vitamin B1 [thiamine] at 10 ug/ml; agar =DIFCO "Purified") containing aminomethylphosphonate (AMPA; 0.2 mM; Sigma) as P source.

MOPS medium is:

10 ml 10× MOPS SALTS
2 ml 0.5 mg/ml Thiamine HCl
1 ml 20% glucose

10× MOPS Salts are:

for 100 ml
40 ml 1M MOPS pH7.4
4 ml 1M Thiamine pH7.4
1 ml 0.01 M FeSO₄·7H₂O
5 ml 1.9 M NH₄Cl
1 ml 0.276 M K₂SO₄
1 ml 0.5 mM CaCl₂
1 ml 0.528 M MgCl₂
10 ml 5M NaCl
1 ml 0.5% L-Methionine
1 ml Micronutrients

Micronutrients are:

3×10⁻⁵M (NH₄)₆Mn₇O₂₄
4×10⁻⁷M H₃BO₄
3×10⁻⁶M CoCl₂
1.6×10⁻⁵M CuSO₄
8×10⁻⁶M MnCl₂
1×10⁻⁶M ZnSO₄

Six individual colonies were picked from this plate after three days incubation at 37° C. and streaked on MOPS complete agar containing either AMPA or methylphosphonate (Alfa) as P source. One colony, designated *E. coli* SR200 Mpu+, was chosen from those that grew equally and uniformly on both phosphonate media.

Chromosomal DNA was prepared from strain LBAA as follows: The cell pellet from a 100 ml L-Broth (Miller, 1972) late log phase culture of LBAA was resuspended in 10 ml of Solution I (Birboim and Doly, 1979). SDS was added to a final concentration of 1% and the suspension was subjected to three freeze-thaw cycles, each consisting of immersion in dry ice for 15 minutes and in water at 70° C. for 10 minutes. The lysate was then extracted four times with equal volumes of phenol:chloroform (1:1; phenol saturated with TE) (TE=10 mM Tris pH8.0; 1.0 mM EDTA) and the phases separated by centrifugation (15000 g; 10 minutes). The ethanol-precipitable material was pelleted from the supernatant by brief centrifugation (8000 g; 5 minutes) following addition of two volumes of ethanol. The pellet was resuspended in 5 ml TE and dialyzed for 16 hours at 4° C. against 2 liters TE. This preparation yielded a 6 ml DNA solution of 150 g/ml.

Partially-restricted DNA was prepared as follows: Three 100 µg aliquot samples of LBAA DNA were treated for 1 hour at 37° C. with restriction endonuclease HindIII at rates of 4, 2 and 1 enzyme unit/µg DNA, respectively. The DNA samples were pooled, made 0.25 mM with EDTA and extracted with equal volume of phenol:chloroform. Following the addition of NaAcetate and ethanol, the DNA was precipitated with two volumes of ethanol and pelleted by centrifugation (12000 g; 10 minutes). The dried DNA pellet

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was resuspended in 500 μ l TE and layered on a 10–40% Sucrose gradient (in 5% increments of 5.5 ml each) in 0.5M NaCl, 50 mM Tris pH8.0, 5 mM EDTA. Following centrifugation for 20 hours at 26,000 rpm in a SW28 rotor, the tubes were punctured and 1 ml fractions collected. Fifteen μ l samples of each third fraction were run on 0.8% agarose gel and the size of the DNA determined by comparison with linearized lambda DNA and HindIII-digested lambda DNA standards. Fractions containing DNA of 25–35 kb fragments were pooled, desalted on AMICON10 columns (7000 rpm; 20° C.; 45 minutes) and concentrated by precipitation. This procedure yielded 50 μ g of LBAA DNA of the required size.

Plasmid pHCT9 (Hohn and Collins, 1980) DNA and a HindIII-phosphatase treated vector was prepared as described elsewhere (Maniatis et al., 1982). The ligation conditions were as follows:

	Vector DNA (HindIII- and calf alkaline phosphatase-treated)	1.6 μ g
20	Size fractionated LBAA	3.75 μ g
	HindIII fragments	
	10x ligation buffer	2.2 μ l
	250 mM Tris-HCl, pH 8.0;	
	100 mM MgCl ₂ ;	
	100 mM Dithiothreitol;	
25	2 mM Spermidine	
	T4 DNA ligase	1.0 μ l
	(Boehringer-Mannheim)	
	(400 units/ μ l)	
	H ₂ O to 22.0 μ l	
	18 hours at 16° C.	

The ligated DNA (4 μ l) was packaged into lambda phage particles (Stratagene; Gigapack Gold) using the manufacturer's procedure.

E. coli SR200 Mpu+, grown overnight in L-Broth (with maltose at 0.2%), was infected with 50 μ l of the packaged DNA. Transformants were selected on MOPS complete agar plus ampicillin and with glyphosate at 0.2 mM as P source.

Aliquot samples were also plated on MOPS (Neidhardt et al., 1974) complete agar plus ampicillin containing Pi at 1 mM to titer the packaged cosmids. Cosmid transformants were isolated on this latter medium at a rate of $\sim 10^5$ per μ g/LBAA HindIII DNA after 2 days at 37° C. Colonies arose on the glyphosate agar from day 3 until day 10 with a final rate of 1 per 200–300 cosmids. Plasmid DNA was prepared from twenty one cosmid transformants from the glyphosate plates. These cosmids fell into at least two classes based on the HindIII restriction pattern of the plasmid DNA. In Class I, all the cosmids had cloned 6.4 and 4.2 kb HindIII restriction fragments in common and in Class II, a ~23 kbp fragment in common. Ten cosmids, representative of the diversity of the cloned fragments, were re-transformed into *E. coli* SR200 Mpu+ and the glyphosate utilization trait verified by selection for growth on MOPS complete agar plus ampicillin plus glyphosate plates. The final cell density achieved by the cultures using glyphosate (0.2 mM in MOPS medium) as a P source was also determined and little difference could be discerned between the different transformants. Transformants were also inoculated into MOPS complete broth with AMPA at 0.1 mM as P source (to ensure the presence of "C-P lyase" activity) and after 24 hours at 37° C. were diluted 100-fold into MOPS complete medium with glyphosate at 0.1 mM and [3-¹⁴C] glyphosate (40,000 cpm/ml). All the cosmid-containing cells degraded glyphosate and generated N-acetylAMPA and N-acetylmethylamine, with no great difference in the rate. The N-acetylAMPA was found in the culture supernatant in these tests. One cosmid from Class I, identified as pMON7468,

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fragment in the BamHI site of pUC118; opposite orientations) were grown in M9-glucose-thiamine- ampicillin broth, with and without the Plac inducer IPTG, harvested in late log phase (Klett 190–220). cell-free lysates of the four cultures were prepared as described above and were assayed for glyphosate-to-AMPA activity with glyphosate at 17 μ M. The highest enzymatic activity was obtained for pMON7469 #1 plus IPTG, where the XhoI site is distal to the Plac, suggesting that the gene(s) were expressed in the BglII-to-XhoI direction.

TABLE II

Glyphosate to AMPA Activity in Cell-Free Lysates of <i>E. coli</i> Transformants		
Clone	IPTG added	Specific Activity pmoles AMPA/min.mg
pMON7469#1	no	<3.0
pMON7469#1	yes	32.0
pMON7469#4	no	<3.0
pMON7469#4	yes	<3.0

The only product observed was AMPA, suggesting that the AMPA acetylating activity that was described earlier had been induced in *E. coli* transformants growing on glyphosate as the P source.

In a later experiment, cell lysates of pMON7469 #1 and pMON7470 (BglII-XhoI 1.8 kb in pUC118; formed from pMON7469 #1 by deletion of the ~700 bp XhoI-SalI fragment) were assayed for glyphosate-to-AMPA activity with glyphosate at 2 mM (Sp. Act. [3-¹⁴C] glyphosate=3.7 mCi/mmol; 0.2 μ Ci/reaction; cultures grown with IPTG in medium) and much higher enzymatic activities were recorded, reflecting the improved assay conditions.

TABLE III

Glyphosate to AMPA Activity in Cell-Free Lysates of <i>E. coli</i> Transformants		
Clone	Specific Activity nmoles AMPA/min.mg	
pMON7469#1	15.04	
pMON7470	7.15	

The proteins encoded by the BglII fragment were determined in vivo using a T7 expression system (Tabor and Richardson, 1985) following cloning of this fragment into the BamHI site in the vector pBlueScript (+) (pMON7471#1, #2; opposite orientations). Test and control plasmids were transformed into *E. coli* K38 containing pGP1-2 (Tabor and Richardson, 1985) and grown at 30° C. in L-broth (2 ml) with ampicillin and kanamycin (100 and 50 μ g/ml, respectively) to a Klett reading of ~50. An aliquot was removed and the cells collected by centrifugation, washed with M9 salts (Miller, 1972) and resuspended in 1 ml M9 medium containing glucose at 0.2%, thiamine at 20 μ g/ml and containing the 18 amino acids at 0.01% (minus cysteine and methionine). Following incubation at 30° C. for 90 minutes, the cultures were transferred to a 42° C. water bath and held there for 15 minutes. Rifampicin (Sigma) was added to 200 μ g/ml and the cultures held at 42° C. for 10 additional minutes and then transferred to 30° C. for 20 minutes. Samples were pulsed with 10 μ Ci of ³⁵S-methionine for 5 minutes at 30° C., the cells collected by centrifugation and suspended in 60–120 μ l cracking buffer (60 mM Tris-HCl 6.8/1% SDS/1% 2-mercaptoethanol/10% glycerol/0.01% bromophenol blue). Aliquot samples were

of AUG₁₂₀ had no effect on glyphosate tolerance while it was abolished by the mutagenesis that introduced the BglII site upstream of AUG₁₈₆. The effects of these mutageneses on the ~45 kd protein were examined by subcloning the mutated sequences into T7 expression vectors using a site in the polylinker of pMON7470 (Kpn I), just upstream of the original BglII site, and the downstream HindIII site. This complete fragment was recloned into pl8UT3T7 (PHARMACIA) KpnI-HindIII and tested in vivo as described above. The ~45 kd protein was still expressed and at comparable levels from both of the "BglII" mutagenized sequences. When the new BglII sites were used as 5' ends (and the downstream HindIII site) for cloning into the pBlueScript BamHI-HindIII sites, the ~45 kd protein was still expressed when the new BglII site upstream of AUG₁₂₀ served as 5' end, but not when that located upstream of AUG₁₈₆ was the 5' end. These data suggest strongly that the AUG₁₂₀ (or some codon located very close to it) is the N-terminus of the glyphosate oxido-reductase protein. The BglII site introduced upstream of the AUG₁₈₆ did not result in a prematurely terminated or highly unstable protein and suggests that the predicted coding sequence changes resulting from this mutagenesis (Val₁₈-Cys₁₉→Arg₁₈-Ala₁₉) had severe effects on the activity of the enzyme.

Further data to confirm the location of the N-terminus were obtained by introducing separately (by mutageneses of pMON7470), an NcoI restriction site recognition sequence (CCATGG for CTATGT; changes the second codon from Serine to Alanine) or an NdeI sequence (CATATG for CCTATG) at AUG₁₂₀ and expressing this ORF using efficient *E. coli* expression vectors. The expression of the NdeI version is outlined here: the NdeI-HindIII fragment, beginning at the putative AUG, was cloned into pMON2123 (NdeI-HindIII) replacing the ompF-IGF-1 fusion fragment (Wong et al., 1988). The resultant clone was introduced into *E. coli* JM101 and the cells induced with nalidixic acid as described (Wong et al., 1988) for 2 hours. The resultant protein was indistinguishable in size from the ~45 kd protein on SDS PAGE and a cell lysate from an induced culture had a glyphosate oxidoreductase specific activity of 12.8 nmoles AMPA/min.mg. When compared in a separate experiment, no differences were observed for the glyphosate oxidoreductase activity when the second codon was Alanine instead of Serine. The structural DNA sequence for the glyphosate oxidoreductase enzyme (SEQ ID NO:4) begins at nucleotide 120 and ends at nucleotide 1415 of the BglII-XhoI fragment of FIG. 2 and the glyphosate oxidoreductase enzyme consists of 431 amino acids (SEQ ID NO:5).

Construction of Glyphosate Oxidoreductase Plant Gene Transformation Vectors

To facilitate the manipulation of the structural glyphosate oxidoreductase gene, the internal EcoRI and NcoI restriction site recognition sequences were removed by sitedirected mutagenesis to substitute the sequence GAATTC for GAATTC and CCACGG for CCATGG, respectively. A glyphosate oxidoreductase coding sequence suitable for introduction into and expression in plant transformation vectors was assembled in the following way: the NcoI ("Met-Ala") N-terminus was combined with the NcoI- and EcoRI-deleted coding sequences, and the C-terminus deleted to the Scal site, in a number of cloning steps using the internal SphI and EcoRV restriction sites. In these steps a BglII site was located immediately upstream of the NcoI site and EcoRI and HindIII sites were located immediately downstream from the stop codon. The sequence of this manipulated glyphosate oxidoreductase gene (SEQ ID NO:6) is shown in FIG. 3. The manipulated glyphosate

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oxidoreductase gene still codes for the wild-type glyphosate oxidoreductase protein. The manipulations do not alter the amino acid sequence of the glyphosate oxidoreductase. This glyphosate oxidoreductase structural sequence (SEQ ID NO:6), as a BglII/NcoI—EcoRI/HindIII fragment of 1321 bp, is readily cloned into an appropriate plant expression cassette. This glyphosate oxidoreductase gene (SEQ ID NO:6) was cloned as a BglII-EcoRI fragment into the plant transformation and expression vector pMON979 to form pMON17073.

Modification and Resynthesis of the Glyphosate Oxidoreductase Gene Sequence

The glyphosate oxidoreductase gene from LBAA contains sequences that could be inimical to high expression of the gene in plants. These sequences include potential polyadenylation sites that are often A+T-rich, a higher G+C% than that frequently found in plant genes (56% versus ~50%), concentrated stretches of G and C residues, and codons that are not used frequently in plant genes. The high G+C% in the glyphosate oxidoreductase gene has a number of potential consequences including the following: a higher usage of G or C than that found in plant genes in the third position in codons, and the potential to form strong hair-pin structures that may affect expression or stability of the RNA. The reduction in the G+C content of the glyphosate oxidoreductase gene, the disruption of stretches of G's and C's, the elimination of potential polyadenylation sequences, and improvements in the codon usage to that used more frequently in plant genes, could result in higher expression of glyphosate oxidoreductase in plants.

In the first phase of this experiment, selected regions of the gene were modified by site-directed mutagenesis. These modifications were directed primarily (but not exclusively) at reducing the G+C% and at breaking up some of the G+C clusters. The manipulated glyphosate oxidoreductase gene was first recloned into the phagemid vector pMON7258 as a NcoI-HindIII fragment to form pMON17014. Single stranded DNA was prepared from a dut ung *E. coli* strain.

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Seven regions of the gene were modified by site-directed mutagenesis using the primers listed in Table IV and the Bio Rad mutagenesis kit (Catalog #170-3576) and following the protocols provided with this kit.

For the sake of clarity, the reverse complement of the actual primers is presented. The base positions, in the sequences presented in FIG. 2 and in FIG. 3, corresponding to the primers are indicated by the first and second set of numbers, respectively.

TABLE IV

Primers to Modify the Glyphosate Oxidoreductase
Gene Coding Sequence

PRIMER 1 (149-210; 38-99)

CGCTGGAGCT GGAATCGTTC GTGTATGCAC TGCTTTGATG CTTCACGTC
GTGGATTCAA AG (SEQ ID NO: 27)
PRIMER 2 (623-687; 512-576)

GCAGATCCTC TCTGCTGATG CTTTGCCTGA TTTCGATCCT AACTTGTCGC
ATGCTTTTAC CAAGG (SEQ ID NO: 28)
PRIMER 3 (792-832; 681-721)

GTCATCGGTT TTGAGACTGA AGGTCGTGCT CTCAAAGCCA T (SEQ ID NO: 29)
PRIMER 4 (833-901; 722-790)

TACAACCACT AACGGTGTTC TGGCTGTGTA TGCAGCTGTT GTTCAGCTG
GTGCACACTC TAAATCACT (SEQ ID NO: 30)
PRIMER 5 (1031-1091; 920-980)

GGAAATGGGT CTCGCTGTTC CTGGTACTGT TGAGTTTGCT GGTCTCACAG
CTGCTCCTAA C (SEQ ID NO: 31)
PRIMER 6 (1179-1246; 1068-1135)

TGGATGGGT TTGCTCTAG CATTCCTGAT TCTCTCCAG TGATTGGTCG
TGCAACTCGT ACACCCGA (SEQ ID NO: 32)
PRIMER 7 (1247-1315; 1136-1204)

CGTAATCTAT GCTTTTGGTC ACGGTCATCT CGGTATGACA GGTGCTCAA
TGACTGCCAC TCTCGTCTC (SEQ ID NO: 33)

The resultant gene (SEQ ID NO:7) was confirmed by sequencing and by the ability to provide comparable glyphosate tolerance levels as the manipulated glyphosate oxidoreductase gene control. This modified gene (SEQ ID NO:7) is referred to as "modified glyphosate oxidoreductase." The G+C% of the glyphosate oxidoreductase gene (SEQ ID NO:6) was reduced from ~56% in the manipulated version to 52% in the modified version (SEQ ID NO:7). A comparison of the manipulated and modified glyphosate oxidoreductase gene is shown in FIG. 3, with the manipulated version on top and the changes introduced to make the modified version on the bottom. This modified glyphosate oxidoreductase gene was cloned as a BglII-EcoRI fragment into a plant expression cassette comprising the En-CaMV35S promoter and the NOS 3' sequences. This cassette was then cloned as a NotI fragment into the pMON886 vector to form pMON17032 (FIG. 5).

A synthetic glyphosate oxidoreductase gene (SEQ ID NO:8) was designed to change as completely as possible those inimical sequences discussed above. In summary, the gene sequence was redesigned to eliminate as much as possible the following sequences or sequence features (while avoiding the introduction of unnecessary restriction sites): stretches of G's and C's of 5 or greater; A+T rich regions (predominantly) that could function as polyadenylation sites or potential RNA destabilization region, and

codons not frequently found in plant genes. A comparison of the manipulated (SEQ ID NO:6) and synthetic (SEQ ID NO:8) glyphosate oxidoreductase genes is presented in FIG. 4, with the manipulated gene (SEQ ID NO:6) on top and the differences introduced into the synthetic gene (SEQ ID NO:8) on the bottom. The G+C% for the synthetic glyphosate oxidoreductase gene is ~51% and the potential to form short, high energy, hair-pin structures is reduced. This synthetic gene was cloned as a BglII-EcoRI fragment into pMON979 to form pMON17065 for introduction into plants.

Expression of Chloroplast Directed Glyphosate Oxidoreductase

The glyphosate target in plants, the 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) enzyme, is located in the chloroplast. Although glyphosate oxidoreductase activity located in the cytoplasm reduces/prevents glyphosate from reaching the chloroplast in the transgenic plant, directing the glyphosate oxidoreductase enzyme to the chloroplast has been found to further minimize the effects of glyphosate on EPSP synthase. Many chloroplast-localized proteins are expressed from nuclear genes as precursors and are targeted to the chloroplast by a chloroplast transit peptide (CTP) that is removed during the import steps. Examples of such chloroplast proteins include the small subunit (SSU) of Ribulose-1,5-bisphosphate carboxylase (RUBISCO), 5-enol-pyruvyl-shikimate-3-phosphate synthase (EPSPS), Ferredoxin, Ferredoxin oxidoreductase, the Light-harvesting-complex protein I and protein II, and Thioredoxin F. It has been demonstrated in vivo and in vitro that non-chloroplast proteins may be targeted to the chloroplast by use of protein fusions with a CTP and that a CTP sequence is sufficient to target a protein to the chloroplast (della-Cioppa et al., 1987).

The glyphosate oxidoreductase protein was targeted to the chloroplast by construction of a fusion between the C-terminus of a CTP and the N-terminus of glyphosate oxidoreductase. In the first example, a specialized CTP, derived from the SSU 1A gene from *Arabidopsis thaliana* (Timko et al., 1988) was used. This CTP (designated CTP1) was constructed by a combination of site-directed mutageneses. The CTP1 structure (SEQ ID NO:9) (FIG. 6) is made up of the SSU 1A CTP (amino acids 1-55), the first 23 amino acids of the mature SSU 1A protein (amino acids 56-78), a serine residue (amino acid 79), a new segment that repeats amino acids 50 to 56 from the SSU 1A CTP and the first two amino acids from the mature protein (amino acids 80-87), and an alanine and methionine residue (amino acids 88 and 89). An NcoI restriction site is located at the 3' end (spans the Met codon) to facilitate the construction of precise fusions to the 5' of glyphosate oxidoreductase or other genes. At a later stage, a Bgl site was introduced upstream of the N terminus of the SSU 1A sequences to facilitate the introduction of the fusions into plant transformation vectors. A fusion was assembled between the CTP1 (SEQ ID NO:9) and the manipulated glyphosate oxidoreductase (SEQ ID NO:6) (through the NcoI site) in the pGEM3zf(+) vector to form pMON17034. This vector may be transcribed in vitro using the SP6 polymerase and the RNA translated with ³⁵S-Methionine to provide material that may be evaluated for import into chloroplasts isolated from *Lactuca sativa* using the methods described hereinafter (della-Cioppa et al., 1986, 1987). This CTP1-glyphosate oxidoreductase fusion was indeed found to be imported into chloroplasts at about 9% efficiency of that of the control, ³⁵S labeled PreEPSPS (pMON6140; della-Cioppa et al., 1986). A CTP1-glyphosate oxidoreductase fusion was then assembled with the synthetic

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glyphosate oxidoreductase gene (SEQ ID NO:8) and this was introduced as a BglII-EcoRI fragment into plant vector pMON979 to form pMON17066 (FIG. 7). Following an intermediate cloning step to acquire more cloning sites, this CTP1-glyphosate oxidoreductase fusion was also cloned as a XbaI-BamHI site into pMON981 to form pMON17138 (FIG. 8).

In the second example, a CTP-glyphosate oxidoreductase fusion was constructed between the *Arabidopsis thaliana* EPSPS (Klee et al., 1987) CTP and the synthetic glyphosate oxidoreductase coding sequences. The *Arabidopsis* CTP was first engineered by site-directed mutagenesis to place a SphI restriction site at the CTP processing site. This mutagenesis replaced the Glu-Lys at this location with Cys-Met. The sequence of this CTP, designated CTP2, (SEQ ID NO:10) is shown in FIG. 9. The NcoI site of the synthetic glyphosate oxidoreductase gene (SEQ ID NO:8) was replaced with a SphI site that spans the Met codon. The second codon was converted to one for leucine in this step also. This change had no apparent effect on the in vivo activity of glyphosate oxidoreductase in *E. coli*. The CTP2-synthetic glyphosate oxidoreductase fusion was cloned into pBlueScript KS(+) and this template was transcribed in vitro using T7 polymerase and the ³⁵S-methionine-labeled material was shown to import into chloroplasts with an efficiency comparable to that for the CTP1-glyphosate oxidoreductase fusion. This CTP2-synthetic glyphosate oxidoreductase fusion was then cloned as a XbaI-BamHI fragment into a plant expression vector to form pMON17164. A structural map of this plasmid is presented in FIG. 12.

The plant vector portion of pMON17164 (FIG. 12) is composed of the following segments. A chimeric kanamycin resistance gene engineered for plant expression to allow selection of the transformed tissue. The chimeric gene consists of the 0.35 Kb cauliflower mosaic virus 35S promoter (P-35S) (Odell et al., 1985), the 0.83 Kb neomycin phosphotransferase typeII gene (KAN), and the 0.26Kb 3' non-translated region of the nopaline synthase gene (NOS 3') (Fraley et al., 1983). A 0.45 Kb ClaI to DraI fragment from the pTi15955 octopine Ti plasmid, which contains the T-DNA left border region (Barker et al., 1983) A 0.75 Kb segment containing the origin of replication from the RK2 plasmid (ori-V) (Stalker et al., 1981) A 3.0 Kb SalI to PstI segment of pBR322 which provides the origin of replication for maintenance in *E. coli* (ori-322) and the bom site for the conjugational transfer into *Agrobacterium tumefaciens* cells. A 0.93 Kb fragment isolated from transposon Tn7 which encodes bacterial spectinomycin/streptomycin resistance (Spc/Str) (Fling et al., 1985), and is a determinant for selection in *E. coli* and *Agrobacterium tumefaciens*. A 0.36 Kb PvuI to BclI fragment from the pTTT37 plasmid, which contains the nopaline-type T-DNA right border region (Fraley et al., 1985). An expression cassette consisting of the 0.6 Kb 35S promoter from the figwort mosaic virus (P-FMV) (Gowda et al., 1989), several unique cloning sites, and the 0.7 Kb 3' nontranslated region of the pea rbcS-E9 gene (E9 3') (Coruzzi et al., 1984, and Morelli et al., 1985). The CTP2-synthetic glyphosate oxidoreductase fusion fragment was cloned into this expression cassette. The introduction of this plasmid into *Agrobacterium* and subsequent plant transformation is described in the Examples to follow.

Those skilled in the art will recognize that various chimeric constructs can be made which utilize the functionality of a particular CTP to import the contiguous glyphosate oxidoreductase enzyme into the plant cell chloroplast. The chloroplast import of the glyphosate oxidoreductase can be determined using the following assay.

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by its ability to utilize glyphosate as a phosphorous source and later shown to contain a putative glyphosate oxidoreductase gene by hybridization with the LBAA glyphosate oxidoreductase gene probe. This gene was cloned initially in a T7 promoter cosmid by screening for glyphosate tolerance in *E. coli* HB101/pGP1-2 (Boyer and Rolland-Dussoix, 1969; Tabor and Richardson, 1985) on M9 medium containing glyphosate at 3 mM. The presence of the glyphosate oxidoreductase gene was first indicated by a positive hybridization signal with the LBAA gene and by its location on a 2.5 kb BglII fragment. This BglII fragment was cloned into the BamHI site in pBlueScript (pMON17183) and expressed from the lac promoter by addition of IPTG. In this experiment a glyphosate oxidoreductase with a specific activity of 53 nmoles/min.mg was obtained, confirming the isolation of the gene by this strategy. The following features have usually been found for these glyphosate oxidoreductase genes: the genes are found (by Southern hybridization using full-length glyphosate oxidoreductase gene probes) on ~2.5 kb BglII fragments, on ~3.5 PstI fragments, contain one EcoRI site within the gene and the genes do not contain a HindIII site. FIG. 13 illustrates some common features of these genes.

The high degree of similarity of glyphosate oxidoreductase genes also suggests another way by which new glyphosate oxidoreductase genes may be cloned. The apparent conservation of regions flanking the genes and the absence of certain restriction sites suggests the use of single-stranded oligonucleotide probes to the flanking regions, containing restriction sites for BglII, HindIII, PstI, BamHI, NdeI, or other suitable cloning sites, and PCR (Polymerase Chain Reaction; see Erlich, 1989, for complete details on PCR and its applications) to amplify a glyphosate oxidoreductase gene fragment suitable for cloning. The flanking sequences for 119 bp upstream (SEQ ID NO:11) of the wild-type (LBAA isolate) glyphosate oxidoreductase gene and for ~290 bp (SEQ ID NO:12) downstream of the gene are provided in FIG. 2.

Using this PCR approach, glyphosate oxidoreductase genes from a number of sources have been isolated. The presence of the glyphosate oxidoreductase activity was confirmed by cloning the glyphosate oxidoreductase gene from chromosomal DNA prepared from *Pseudomonas* sp. strain LBr (Jacob et al., 1988) and using primers homologous to the N- and C-termini of the LBAA glyphosate oxidoreductase gene and containing the following suitable restriction cloning sites: 5'-GAGAGACTGT CGACTC-CGCG GGAGCATCAT ATG-3' (SEQ ID NO:13) and 5'-GAACGAATCC AAGCTTCTCA CGACCGCGTA AGTAC-3' (SEQ ID NO:14). Cyclotherm parameters used for these PCR reactions is as follows:

Denature at 94° C. for 1 minute;
 Anneal at 60° C. for 2 minutes;
 Polymerize at 72° C. for 3 minutes.
 30 cycles, no autoextension, linked to 4° C. incubation.
 The expected ~1.3 kb PCR produced was generated and following digestion with NdeI and HindIII, this fragment was cloned into pMON2123 for expression of the encoded enzyme. The glyphosate oxidoreductase activity was measured as described above and the K_m for glyphosate was similar to that for enzymes from LBAA which is presented supra.

source of glyphosate oxidoreductase gene	K_m (glyphosate: mM)
<i>Pseudomonas</i> sp. strain L.Br	25

Bacteria isolated from glyphosate process waste stream treatment facilities may also be capable of converting glyphosate to AMPA. *Pseudomonas* strains LBAA and L.Br are two such examples. Such bacteria may also be isolated de novo from these waste treatment facilities.

A population of bacteria was isolated from a fixed-bed immobilized cell column, which employed Mannville R-635 diatomaceous earth beads, by plating on Tryptone Soy Agar (Difco), containing cycloheximide at 100 ug/ml, and incubating at 28° C. The column had been run for three months on a waste-water feed from the Monsanto Company's Luling, MS, glyphosate production plant. The column contained 50 mg/ml glyphosate and NH_3 as NH_4Cl . Total organic carbon was 300 mg/ml and BOD's (Biological Oxygen Demand—a measure of "soft" carbon availability) was less than 30 mg/ml. This treatment column has been described (Heitkamp et al., 1990). One of the predominant members of this population, identified as *Agrobacterium* sp. strain T10, was found to also grow in minimal broth in which the sole carbon source provided was glyphosate at 10 mM (this broth was made up as for DF medium but with glyphosate substituting for the glucose, gluconate and citrate). Chromosomal DNA was prepared from this isolate and subjected to the same PCR procedure and with the same primers as described above for the strain L.Br. A fragment of the correct size was generated and cloned into the *E. coli* expression vector. The glyphosate oxidoreductase activity was assayed and the K_m for glyphosate also determined:

source of gene	K_m (glyphosate: mM)
<i>Agrobacterium</i> sp. strain T10	28

Glyphosate-to-AMPA conversion has been reported for many different soils (see Malik et al., 1989 for a review) and a number of procedures are available for the extraction of total DNA from mixed environment samples such as soil (Holben et al., 1988; Steffan and Atlas, 1988; Tsai and Olson, 1991), indicating the possibility of cloning glyphosate oxidoreductase genes without having to first isolate such a degrading microorganism. Of course, the procedure described for the cloning of the glyphosate oxidoreductase genes, based on the conferring of a glyphosate utilization ability or glyphosate tolerance on *E. coli*, provides a scheme by which other glyphosate oxidoreductase genes and other glyphosate metabolizing genes may be cloned, without relying on the homology determined for the glyphosate oxidoreductase gene described here. It is possible also to enrich for glyphosate degrading bacteria, for example, by the repeated application of glyphosate to a patch of soil (Quinn et al., 1988; Talbot et al., 1984). This enrichment step might be used to increase the ease with which glyphosate oxidoreductase genes are recovered from soil or other environments.

Evidence for the presence of the glyphosate oxidoreductase gene in soil bacteria and a procedure for the isolation of such genes is outlined in the following: A population of suitable bacteria was enriched for selection of bacteria capable of growing in liquid media with glyphosate (at 10 mM) as a source of carbon (This medium is made up as

described for the Dworkin-Foster medium but with the omission of the carbon sources and with Pi as a source of P). The inoculum was provided by extracting soil (from a recently harvested soybean field in Jerseyville, Ill.) and the population selected by successive culturing in the medium described above at 28° C. (cycloheximide was included at 100 µg/ml to prevent growth of fungi). Upon plating on L-agar medium, 5 colony types were identified. Chromosomal DNA was prepared from 2 ml L-broth cultures of these isolates and the presence of the glyphosate oxidoreductase gene was probed using PCR screening. Using the primers GCCGAGATGACCGTGGCCGAAAGC (SEQ ID NO:15) and GGGAATGCCGGATGCTTCAACGGC (SEQ ID NO:16), a DNA fragment of the predicted size was obtained with the chromosomal DNA from one of the isolates (designated S3). The PCR conditions used were as follows: 1 minute at 94° C.; 2 minutes at 40° C.; 3 minutes at 72° C.; 35 cycles. The DNA fragment generated in this way is used as a probe (following radiolabeling) to isolate the S3 glyphosate oxidoreductase gene candidate from a cosmid bank constructed as described for LBAA DNA and greatly facilitates the isolation of other glyphosate oxidoreductase genes. The primers used are homologous to internal sequences in the LBAA glyphosate oxidoreductase gene. The PCR conditions employed allow a fair degree of mismatch in the primers and the result suggests that the glyphosate oxidoreductase gene from S3 may not be as closely related to the other glyphosate oxidoreductase genes that were successfully isolated using the primers to the N- and C-termini of the LBAA gene.

A variety of procedures are available for the isolation of genes. Some of these procedures are based on the knowledge of gene function that allow the design of phenotypic screens to aid in the isolation. Others are based on at least partial DNA sequence information that allow the use of probes or primers with partial or complete homology, or are based on the use of antibodies that detect the gene product. All of these options may be applied to the cloning of glyphosate oxidoreductase genes.

40 Improvement of the Kinetic Properties of Glyphosate Oxidoreductase

Prior examples of engineered herbicide resistance by enzymatic inactivation of the herbicide have utilized enzymes with an ability to bind and metabolize the herbicides much more efficiently than glyphosate oxidoreductase metabolizes glyphosate. The glyphosate oxidoreductase enzyme has a K for glyphosate of 20–30 mM and, as a result, the reaction rate for the degradation of glyphosate may be enhanced for optimal efficiency in transgenic plants by either lowering the Km or by raising the V.

Random mutagenesis techniques coupled with appropriate selections and/or screens are powerful tools which have been used successfully to generate large numbers of mutagenized gene sequences and potential variants. The same approaches may be used to isolate and to identify glyphosate oxidoreductase variants with improved glyphosate degradation efficiency. The mutagenesis techniques that may be employed include chemical mutagenesis of bacterial cultures containing the gene of interest or of purified DNA containing this gene and PCR methods used to generate copies of the gene (or portions of it) under conditions that favor misincorporation of nucleotides (errors) into the new strand. An example of such a condition would be carrying out the PCR reaction in the presence of Mn++.

Appropriate in vivo screens for improved variants following the mutagenesis could include those for improved glyphosate tolerance in *E. coli* or increased growth on

was recloned into the expression vector portion and this phenotype verified. All kinetic analysis was performed on crude *E. coli* lysates. Putative glyphosate oxidoreductase variant proteins were overexpressed after subcloning the NcoI/HindIII variant glyphosate oxidoreductase gene into PrecA-gene10L expression vector. For overexpression in PrecA-gene10L constructs, GB993 cells containing the vector were induced at a Klett=110–120 in M9 minimal medium with 50 µg/ml nalidixic acid and allowed to grow for 2.5 hours at 37° C. with vigorous shaking. Cells were harvested by centrifugation at 4000 g, 5 minutes at 4° C., and resuspended in 100 mM Tris-HCl, pH 7.1, 1 mM EDTA, 35 mM KCl, 20% glycerol, and 1 mM benzamidine at 3 ml/g cell pellet. Lysates were prepared by breaking the cells in a French press, twice, at 1000 psi. Insoluble debris was removed by centrifugation at 12000 g, 15 minutes at 4° C., and the supernatant was de-salted by passing over a PD-10 column (Sephadex G-25, Pharmacia). The void volume fraction was used as the source of enzyme for kinetic analysis. Protein concentrations were determined using the Bio-Rad protein dye-binding assay. Time and enzyme concentration courses were performed to determine linear ranges. The enzyme assay was performed as follows: lysate and glyphosate oxidoreductase mix (final concentration =0.1M MOPS, 0.01M Tricine, pH 7.4, 0.01 mM FAD, 10 mM MgCl₂) in a 100 µl reaction were pre-incubated at 30° C. for 2 minutes prior to the addition of glyphosate (analytical grade stock prepared in water adjusted to pH 7.0 with NaOH). Ten minutes was determined to be the optimal time for the enzyme assay using 10 µg lysate. After 10 minutes at 30° C. with shaking, 0.25 ml dinitrophenylhydrazine (DNPH) reagent (0.5 mg/ml in 0.5M HCl) was added and the reaction was allowed to proceed for an additional 5 minutes at 30° C. with shaking. A 1.5M NaOH solution (400 µl) was then added to the assay mix, and the reaction was continued for 5 minutes at 30° C. with shaking. Enzyme activity was determined from the amount of glyoxylate-DNPH adduct formed by measuring A₅₂₀ against a standard of glyoxylate. Enzyme assays are performed in duplicate on at least two different single colony isolates of a putative glyphosate oxidoreductase variant. To determine K_m and V_{max}, enzyme assays were performed over a (0.2–2.0)×K_m range of glyphosate concentrations. The K_m and V_{max} were determined from Lineweaver Burk, Eadie-Hofstee and hyperbolic kinetic plots. V_{max} was estimated after determining the amount of immunoreactive glyphosate oxidoreductase protein in lysates by immunoblot analysis as described below. Immunoblot analysis was performed following SDS-PAGE and transfer of protein from the gel to nitrocellulose at 500 mA in a Hoeffer transfer apparatus in 25 mM Tris-HCl, 192 mM glycine containing 0.1% SDS and 25% methanol for 1–2 hours. After transfer, the nitrocellulose was incubated with 50 mM Tris-HCl, pH7.5, 0.9% NaCl, 0.01% Tween 20, 0.02% NaN₃ containing 2% bovine serum albumin at room temperature with shaking for at least 30 minutes. After blocking, the same buffer containing a 1:25,000 dilution of goat anti-glyphosate oxidoreductase antiserum was added and the filter was allowed to shake at room temperature for 45 minutes. After incubation with primary glyphosate oxidoreductase antibody, the filter was washed for 45 minutes in buffer without antibody; buffer containing a 1:5000 dilution of rabbit anti-goat alkaline phosphatase-conjugated second antibody (from Pierce) was added and the filter was incubated for 45 minutes at room temperature with shaking. The filter was then washed in buffer without antibody for 30 minutes prior to addition of NBT and BCIP (Promega) to allow color development. Immunoreactive

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glyphosate oxidoreductase protein was also quantitated by dot blotting the lysate onto nitrocellulose and then processing the filter as described above, except that 125 I-Protein G was used for detection. The amount of glyphosate oxidoreductase protein in lysates was determined by counting the dot and comparing the amount of radioactivity against a glyphosate oxidoreductase protein standard. One variant, v.247, showed a 3–4-fold higher specific activity for glyphosate oxidoreductase at 25 mM glyphosate and the immunoblot analysis indicated that this was not due to an elevated glyphosate oxidoreductase protein level. Subsequent assays indicated that this variant had a 10-fold lower K_m for glyphosate than the wild type glyphosate oxidoreductase. In a similar manner the K_m for IDA was also determined and these data are presented below.

Kinetic analysis of glyphosate oxidoreductase variants:

Variant	app K_m (mM)		app V_m (U/mg)		V_m/K_m	
	Glyp	IDA	Glyp	IDA	Glyp	IDA
wild type	27.0	2.8	0.8	0.5	.03	18
v.247	2.6	0.7	0.6	0.7	.23	1.0

The glyphosate oxidoreductase gene from v.247 was sequenced (SEQ ID NO:17) and five nucleotide changes were found. These changes are described in the following as they relate to the codons: GCT to GCC (codon 43), no amino acid change; AGC to GGC (codon 84), Ser to Gly; AAG to AGG (codon 153), Lys to Arg; CAC to CGC (codon 334), His to Arg, and CCA to CCG (codon 362), no amino acid change. The amino acid sequence of the glyphosate oxidoreductase gene from v.247 is presented as SEQ ID NO:18. The importance of these different amino acid changes was determined initially by recloning the altered regions into wild type glyphosate oxidoreductase and determining the effect on glyphosate oxidoreductase activity and kinetics. This was accomplished by recloning the NcoI-NheI fragment (contains codon 84), the NheI-ApaI fragment (contains codon 153), and the ApaI-HindIII fragment (contains codon 334), separately into the wild type gene. These glyphosate oxidoreductase genes were then expressed and the kinetic analyses performed. The data are presented below and indicate that the change that occurred in the ApaI-HindIII fragment (contains codon 334) was responsible solely for the alteration in the enzyme.

Kinetic analysis of domain switches

Clone	app K_m (mM)	app V_m (U/mg)	V_m/K_m
wt (w1w2w3*)	28.4	0.65	0.022
v.247 (v1v2v3**)	2.1	0.72	0.34
w1v2w3	23.5	0.62	0.026
w1v2v3	2.1	0.6	0.28
w1w2v3	2.0	0.75	0.375
v1w2v3	2.6	0.55	0.21
v1w2w3	28.0	0.75	0.027
v1v2w3	26.7	0.55	0.021

*w1 = SER84; w2 = LYS153; w3 = HIS334

**v1 = GLY84; v2 = ARG153; v3 = ARG334

This result was confirmed and extended by repeating the His to Arg change at codon 334 and introducing other specific changes at this residue by site-directed mutageneses. The primers used are listed in the following: Arg - CGTTCTCTAC ACTCGTGCTC GTAAGTTGC (SEQ ID NO:19); Lys - CGTTCTCTAC ACTAAGGCTC GTAAGT-

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TGC (SEQ ID NO:20); Gin - CGTTCCTCTAC ACT-
CAAGCTC GTAAGTTGC (SEQ ID NO:21); and Ala -
CGTTCCTCTAC ACTGCTGCTC GTAAGTTGC (SEQ ID
NO:22) (These sequences are the antisense to those actually
used). The presence of these changes was confirmed by
sequencing the mutagenized glyphosate oxidoreductase
genes and a kinetic analysis of the expressed glyphosate
oxidoreductase enzymes was performed. The data are pre-
sented in the following and show that a number of substi-
tutions are possible at this position and which result in an
enzyme with altered kinetic properties.

Kinetic analysis of glyphosate oxidoreductase variants.

Variant	app K_m (mM)		app V_m (U/mg)		V_m/K_m	
	Glyp	IDA	Glyp	IDA	Glyp	IDA
wild type	27.0	2.8	0.8	0.5	03	.18
v 247	2.6	0.7	0.6	0.7	23	1.0
ARG 334	2.6	0.5	0.6	0.6	23	1.2
LYS 334	9.9	1.3	0.7	0.8	.07	.62
GLN 334	19.6	3.5	0.6	0.7	03	20
ALA 334	26.7	3.5	0.2	0.2	007	.057

Additional mutageneses were performed to change the
His334 residue to other amino acids. The primers to accom-
plish this and the new codon are listed in the following: Trp
- CTCTACACTTGGGCTCGTAAGCTTCTTCCAGC
(SEQ ID NO:23); le - CTCTACACTATCGCTCGTAAGCT-
TCTTCCAGC (SEQ ID NO:24); Lau - CTCTACACTCTG-
GCTCGTAAGCTTCTTCCAGC (SEQ ID NO:25); and Glu
- CTCTACACTGAAGCTCGTAAGCTTCTTCCAGC
(SEQ ID NO:26) (These sequences are the antisense of those
actually used; these primers also add a "silent" HindIII that
facilitates the identification of the mutagenized progeny
from the population). The GLU334 variant retains substan-
tial glyphosate oxidoreductase activity, while the TRP334,
ILE334, and LEU334 variants retain much less activity.

From the first generation variants, those with the highest
 k_{cat}/K_m ratio are preferably subjected to a second round of
mutagenesis followed by subsequent screening and analysis.
An alternative approach would be to construct second gen-
eration glyphosate oxidoreductase variants by combining
single point mutations identified in the first generation
variants.

PLANT TRANSFORMATION

Plants which can be made glyphosate tolerant by practice
of the present invention include, but are not limited to,
soybean, cotton, corn, canola, oil seed rape, flax, sugarbeet,
sunflower, potato, tobacco, tomato, wheat, rice, alfalfa,
lettuce, apple, poplar and pine.

A double-stranded DNA molecule of the present invention
("chimeric gene") can be inserted into the genome of a plant
by any suitable method. Suitable plant transformation vec-
tors include those derived from a Ti plasmid of *Agrobacter-
ium tumefaciens*, as well as those disclosed, e.g., by
Herrera-Estrella (1983), Bevan (1984), Klee (1985) and
EPO publication 120,516 (Schilperoort et al.). In addition to
plant transformation vectors derived from the Ti or root-
inducing (Ri) plasmids of *Agrobacterium*, alternative meth-
ods can be used to insert the DNA constructs of this
invention into plant cells. Such methods may involve, for
example, the use of liposomes, electroporation, chemicals
that increase free DNA uptake, free DNA delivery via
microprojectile bombardment, and transformation using
viruses or pollen.

The pMON979 plant transformation/expression vector was derived from pMON886 (described below) by replacing the neomycin phosphotransferase typeII (KAN) gene in pMON886 with the 0.89 kb fragment containing the bacterial gentamicin-3-N-acetyltransferase type III (AAC(3)-III) gene (Hayford et al., 1988). The chimeric P-35S/AAC(3)-III/NOS 3' gene encodes gentamicin resistance which permits selection of transformed plant cells. pMON979 also contains a 0.95 kb expression cassette consisting of the enhanced CaMV 35S promoter (Kay et al., 1987), several unique restriction sites, and the NOS 3' end (P-En-CaMV35S/NOS 3'). The rest of the pMON979 DNA segments are exactly the same as in pMON886.

Plasmid pMON886 is made up of the following segments of DNA. The first is a 0.93 kb A v a I to engineered-EcoRV fragment isolated from transposon Tn7 that encodes bacterial spectinomycin/streptomycin resistance (Spc/Str), which is a determinant for selection in *E. coli* and *Agrobacterium tumefaciens*. This is joined to the 1.61 kb segment of DNA encoding a chimeric kanamycin resistance which permits selection of transformed plant cells. The chimeric gene (P-35S/KAN/NOS 3') consists of the cauliflower mosaic virus (CaMV) 35S promoter, the neomycin phosphotransferase typeII (KAN) gene, and the 3' -nontranslated region of the nopaline synthase gene (NOS 3') (Fraley et al., 1983). The next segment is the 0.75 kb oriV containing the origin of replication from the RK2 plasmid. It is joined to the 3.1 kb SalI to PvuI segment of pBR322 (ori322) which provides the origin of replication for maintenance in *E. coli* and the bom site for the conjugational transfer into the *Agrobacterium tumefaciens* cells. The next segment is the 0.36 kb PvuI to BclI from pTIT37 that carries the nopaline-type T-DNA right border (Fraley et al., 1985).

The pMON981 plasmid contains the following DNA segments: the 0.93 kb fragment isolated from transposon Tn7 encoding bacterial spectinomycin/streptomycin resistance [Spc/Str; a determinant for selection in *E. coli* and *Agrobacterium tumefaciens* (Fling et al., 1985)]; the chimeric kanamycin resistance gene engineered for plant expression to allow selection of the transformed tissue, consisting of the 0.35 kb cauliflower mosaic virus 35S promoter (P-35S) (Odell et al., 1985), the 0.83 kb neomycin phosphotransferase typeII gene (KAN), and the 0.26 kb 3' -nontranslated region of the nopaline synthase gene (NOS 3') (Fraley et al., 1983); the 0.75 kb origin of replication from the RK2 plasmid (oriV) (Stalker et al., 1981); the 3.1 kb SalI to PvuI segment of pBR322 which provides the origin of replication for maintenance in *E. coli* (ori-322) and the bom site for the conjugational transfer into the *Agrobacterium tumefaciens* cells, and the 0.36 kb PvuI to BclI fragment from the pTIT37 plasmid containing the nopaline-type T-DNA right border region (Fraley et al., 1985). The expression cassette consists of the 0.6 kb 35S promoter from the figwort mosaic virus (P-FMV) (Gowda et al., 1989) and the 0.7 kb 3' non-translated region of the pea rbcS-E9 gene (E9 3') (Coruzzi et al., 1984, and Morelli et al., 1985). The 0.6 kb SspI fragment containing the FMV35S promoter (FIG. 1) was engineered to place suitable cloning sites downstream of the transcriptional start site.

The plant vector was mobilized into the ABI *Agrobacterium* strain. The ABI strain is the A208 *Agrobacterium tumefaciens* carrying the disarmed Ti plasmid pTiC58 (pMP90RK) (Koncz and Schell, 1986). The Ti plasmid does not carry the T-DNA phytohormone genes and the strain is therefore unable to cause the crown gall disease. Mating of the plant vector into ABI was done by the triparental conjugation system using the helper plasmid pRK2013

(Ditta et al., 1980). When the plant tissue is incubated with the ABL:plant vector conjugate, the vector is transferred to the plant cells by the vir functions encoded by the disarmed pTiC58 plasmid. The vector opens at the T-DNA right border region, and the entire plant vector sequence may be inserted into the host plant chromosome. The pTiC58 Ti plasmid does not transfer to the plant cells but remains in the *Agrobacterium*.

PLANT REGENERATION

When adequate production of the glyphosate oxidoreductase activity is achieved in transformed cells (or protoplasts), the cells (or protoplasts) are regenerated into whole plants. Choice of methodology for the regeneration step is not critical, with suitable protocols being available for hosts from Leguminosae (alfalfa, soybean, clover, etc.), Umbelliferae (carrot, celery, parsnip), Cruciferae (cabbage, radish, rapeseed, etc.), Cucurbitaceae (melons and cucumber), Gramineae (wheat, rice, corn, etc.), Solanaceae (potato, tobacco, tomato, peppers) and various floral crops. See, e.g., Ammirato, 1984; Shimamoto, 1989; Fromm, 1990; Vasil, 1990.

The following examples are provided to better elucidate the practice of the present invention and should not be interpreted in any way to limit the scope of the present invention. Those skilled in the art will recognize that various modifications, truncations, etc. can be made to the methods and genes described herein while not departing from the spirit and scope of the present invention.

EXAMPLES

Expression, Activity and Phenotype of Glyphosate Oxidoreductase in Transformed Plants

The transformation, expression and activity of glyphosate oxidoreductase, and the glyphosate tolerance phenotype imparted to the plants by the glyphosate oxidoreductase genes, introduced into *Nicotiana tabacum* cv. "Samsun" and/or *Brassica napus* cv. Westar using the vectors pMON17073, pMON17032, pMON17065, pMON17066, pMON17138, and pMON17164, is described in the following exemplary embodiments. Initial data in tobacco on the expression of the manipulated glyphosate oxidoreductase gene (SEQ ID NO:6) under the control of the En-CaMV35S promoter (see data on pMON17073 in Tables VIII and IX, for example) indicated only low levels of expression of glyphosate oxidoreductase. The transcription of the gene was confirmed in the case of 3-4 plants by Northern and S1 analysis but no glyphosate oxidoreductase protein could be detected (limit of detection in that assay was ~0.01% expression level). Analysis of R_0 plants following spray with 0.4 lb/acre (approximately 0.448 kg/ha) glyphosate also showed only low levels of tolerance. Modification of the gene sequence (as described herein) resulted in improved expression in tobacco, as did the use of the FMV promoter and the use of a CTP fusion to the glyphosate oxidoreductase gene. For these reasons the majority of the data presented comes from transgenic plants derived using vectors containing these improved glyphosate oxidoreductase constructs. One set of experiments with the modified glyphosate oxidoreductase vector pMON17032 are presented in example 1 and a study of manipulated glyphosate oxidoreductase, synthetic glyphosate oxidoreductase, and CTP1-synthetic glyphosate oxidoreductase is presented in example 2. The transformation and expression of glyphosate oxidoreductase in canola is described in example 3.

Example 1

The tobacco leaf disc transformation protocol employs healthy leaf tissue about 1 month old. After a 15-20 minute

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surface sterilization with 10% Clorox plus a surfactant, the leaves were rinsed 3 times in sterile water. Using a sterile paper punch, leaf discs are punched and placed upside down on MS104 media (MS salts 4.3 g/l, sucrose 30 g/l, B5 vitamins 50× 2 ml/l, NAA 0.1 mg/l, and BA 1.0 mg/l) for a 1 day preculture.

The discs were then inoculated with an overnight culture of disarmed *Agrobacterium* ABI containing the subject vector that had been diluted 1/5 (ie: about 0.6 OD). The inoculation was done by placing the discs in centrifuge tubes with the culture. After 30 to 60 seconds, the liquid was drained off and the discs were blotted between sterile filter paper. The discs were then placed upside down on MS104 feeder plates with a filter disc to co-culture.

After 2–3 days of co-culture, the discs were transferred, still upside down, to selection plates with MS104 media. After 2–3 weeks, callus formed, and individual clumps were separated from the leaf discs. Shoots were cleanly cut from the callus when they were large enough to distinguish from stems. The shoots were placed on hormone-free rooting media (MSO: MS salts 4.3 g/l, sucrose 30 g/l, and B5 vitamins 50×2 ml/l) with selection. Roots formed in 1–2 weeks. Any leaf callus assays are preferably done on rooted shoots while still sterile. Rooted shoots were placed in soil and were kept in a high humidity environment (ie: plastic containers or bags). The shoots were hardened off by gradually exposing them to ambient humidity conditions.

A total of 45 Kanamycin resistant pMON17032 tobacco lines were examined (Table V).

TABLE V

Expression of Modified Glyphosate Oxidoreductase Gene in Tobacco (R1 Transgenics of pMON17032)					
# Plants	Glyphosate Recalling (0.5 mM glyphosate)			Western Analysis of Plants ²	
	+	+/-	-	+	-
45	0	11	34	24	21

* + means 0.5–2 ng/50 µg protein
– means <0.5 ng/50 µg protein

Leaf recalling on plant tissue culture media indicated a low level of glyphosate tolerance (rated as a +/--phenotype) for at least 11 of these lines. At least 24 of these lines expressed a detectable level of glyphosate oxidoreductase in the range of 0.5 to 2 ng per 50 µg of extractable protein. The glyphosate tolerance displayed in the leaf recalling assay and the higher glyphosate oxidoreductase expression level indicate that the changes made to the glyphosate oxidoreductase coding sequences to make the modified glyphosate oxidoreductase gene (SEQ ID NO:7) had a marked effect on the ability of this gene to be expressed in plants. This same effect could also then be achieved by expressing the manipulated glyphosate oxidoreductase gene (SEQ ID NO:6) using stronger plant promoters, using better 3' polyadenylation signal sequences, optimizing the sequences around the initiation codon for ribosome loading and translation initiation, or by combinations of these or other expression or regulatory sequences or factors. The R1 progeny of a number of these lines, including those with the highest glyphosate oxidoreductase expression level (#'s 18854 and 18848) were sprayed with glyphosate at rates of 0.4 and 1.0 lb/acre (0.448 and 1.12 kg/ha, respectively) and vegetative performance rated over a period of four weeks (Table VI).

TABLE VI

Tobacco Spray Data for pMON17032 R1 Plants				
Line	Rate kg/ha	Vegetative Score*		
		7 Days	14 Days	28 Days
18860	0.448	3	3	3
	1.12	1	1	2
18842	0.448	4	6	8
	1.12	2	3	6
18848	0.448	3	4	8
	1.12	2	2	6
18854	0.448	4	7	9
	1.12	2	5	8
18858	0.448	3	4	6
	1.12	1	2	4
18885	0.448	4	5	8
	1.12	2	1	2
18890	0.448	3	6	7
	1.12	1	2	3
Samsun	0.448	1	1	2
	1.12	1	1	0

*Vegetative Score

0 = Dead

10 = No detectable effect

Following an initial lag, and especially for those plants expressing the highest levels of glyphosate oxidoreductase, these lines showed vegetative glyphosate tolerance at both spray rates (that improved with time). Glyphosate oxidoreductase enzyme activity was determined for two of the pMON17032 lines (#'s 18858 and 18881). Leaf tissue (1 g) was harvested, frozen in liquid N₂, and stored at -80° C. prior to extraction. For extraction, leaf tissue was pulverized in a mortar and pestle with liquid N₂. To the powdered leaf tissue was then added 1 ml extraction buffer (100 mM TrisCl, pH 7.4, 1 mM EDTA, 20% glycerol, 35 mM KCl, 1 mM benzamidine HCl, 5 mM Na ascorbate, 5 mM dithiothreitol, and 1 mg/ml bovine serum albumin, 4° C.), and the sample was further ground for 1 minute. The resulting mixture was centrifuged for 5 minutes (high speed, Eppendorf) and the supernatant was treated with a saturated ammonium sulfate solution to give 70% final saturation (2.33 ml saturated solution/ml extract). The precipitated protein was collected by centrifugation as above, and the pellet was resuspended in 0.4 ml of extraction buffer. After centrifuging again to remove particulate matter, the sample was desalted using Sephadex G50 contained in a 1 ml syringe, equilibrated with extraction buffer, according to the method of Penefsky (1979). The desalted plant extracts were stored on ice, and protein concentrations were determined by the method of Bradford (1976). Glyphosate oxidoreductase reactions were carried out in duplicate for 60 minutes at 30° C. in an assay mixture of 0.1 MOPS/0.01 tricine buffer, pH 7.4, containing 10 mM MgCl₂, 0.01 mM flavin adenine dinucleotide (FAD, Sigma), and 1 mM ubiquinone Qo, (Sigma). Plant extracts (75 µl) were preincubated in the assay mixture for 2 minutes, and reactions were then initiated by adding iminodiacetic acid (IDA, 20 µl) substrate to a final concentration of 50 mM (total assay volume was 0.2 ml). Reactions were quenched and derivatized as described below. Control reactions omitting IDA and omitting plant extract were also performed. Glyoxylate detection was carried out using 2,4-dinitrophenylhydrazine (2,4-DNPH) derivatization and reverse phase high performance liquid chromatography (HPLC), using a modification of the method of Qureshi et al. (1982). Glyphosate oxidoreductase reactions (0.2 ml) were quenched with 0.25 ml of DNPH reagent (0.5 mg/ml DNPH [Aldrich] in 0.5M HCl) and allowed to derivative for 5 minutes at 25° C. The samples were then extracted with ethyl acetate (2-0.3ml) and the

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combined ethyl acetate extracts were extracted with 10% Na_2CO_3 (0.3 ml). The Na_2CO_3 phase was then washed once with ethyl acetate (0.2 ml) and the Na_2CO_3 phase injected (100 μl) on a Beckman Ultrasphere C18 IP HPLC column (5 μA , 4.6 mm \times 25 cm) using an LKB GTI binary HPLC system with a Waters 990 photodiode array UVN15 HPLC detector, via a Waters WISP HPLC autoinjector. The isocratic mobile phase was methanol-water-acetic acid (60:38.5:1.5) with 5 mM tetrabutylammonium phosphate (Pierce). The DNPH-glyoxylate peak (retention time =6.7 minutes) was detected at 365 nm and compared to a glyoxylate standard (Sigma, 20 μM in 0.2 ml) derivatized in exactly the same manner.

TABLE VII

Glyphosate oxidoreductase Activity of Transgenic Tobacco Plants	
Plant	Specific Activity nmol/min mg
Samsun	0 (not detectable)
18881	0.039
18858	0.018

Example 2

A series of transformed tobacco lines were derived using the "isogenic" glyphosate oxidoreductase vectors pMON17073 (manipulated glyphosate oxidoreductase) (SEQ ID NO:6), pMON17065 (synthetic glyphosate oxidoreductase) (SEQ ID NO:8), and pMON17066 (CTP1-synthetic glyphosate oxidoreductase). By Western analysis (see Table VIII below) of a number of these lines, the manipulated glyphosate oxidoreductase plants were found to express up to ~0.5 ng glyphosate oxidoreductase per 50 μg plant protein, the synthetic glyphosate oxidoreductase at levels from ~0.5 – 2 ng per 50 μg , and at levels from ~2 – 20 ng per 50 μg for the CTP1-synthetic glyphosate oxidoreductase plants.

TABLE VIII

Glyphosate Oxidoreductase Expression in Tobacco		
Construct	Plant #	Western Rating
pMON17073 (manipulated)	21270	0
	21281	0
	21286	1
	21929	1
pMON17066 (CTP1-synthetic)	21237	1
	21830	0
	21845	3
	21872	3
	21889	1
pMON17065 (synthetic)	21891	0
	21199	0
	21208	2
	21211	2
	21217	0
	21218	2
	21792	1
	21795	0
	21811	2

Western rating scale per 50 μg of protein:

0 - no detectable glyphosate oxidoreductase

1 - <0.5 ng

2 - 0.5 ng-2 ng

3 - >2 ng

A number of primary transformants R_0 lines, expressing manipulated or synthetic glyphosate oxidoreductase or CTP1-synthetic glyphosate oxidoreductase, were sprayed

with glyphosate at 0.4 lb/acre (0.448 kg/ha) and rated as before.

TABLE IX

Glyphosate Spray Data: pMON17066 (CTP1-Glyphosate Oxidoreductase) Tobacco (R ₁ plants)					
Vegetative Score#					
(Spray Rate = 0.4 lb/acre) (0.448 kg/ha)					
Line	Western Rating	7	14	28	(days after spray)
Control A	0	3	0	0	no detectable
Control B	0	3	1	0	glyphosate
Control C	0	3	1	1	oxidoreductase
22933	1	3	1	0	(pMON17073)
22741	2	2	1	9	(pMON17065)
22810	3	3	4	6	(pMON17066)
22825	1	2	1	1	(pMON17066)
22822	3	10	10	10	(pMON17066)
22844	3	10	10	10	(pMON17066)
22854	3	9	10	10	(pMON17066)
22860	3	8	10	10	(pMON17066)
22880	1	3	2	9	(pMON17066)
22881	2	2	0	0	(pMON17066)
22886	3	9	10	10	(pMON17066)
22887	3	9	10	10	(pMON17066)

Western rating scale (per 50 µg protein)
 0 = no detectable glyphosate oxidoreductase
 1 = <0.5 ng
 2 = 0.5-2 ng
 3 = >2 ng

Vegetative score:
 0 = dead;
 10 = no detectable effect

The synthetic glyphosate oxidoreductase line displayed a response similar to that noted for the modified glyphosate oxidoreductase R₁ plants, in that there was some immediate glyphosate effects that were overcome with time, through the metabolism of the herbicide by glyphosate oxidoreductase to the derivatives AMPA and glyoxylate. Since the target of glyphosate (EPSP synthase) is located in the chloroplast, the activity of glyphosate oxidoreductase must be reducing the level of glyphosate within this organelle by removing the herbicide before it reaches the chloroplast. The CTP1-synthetic glyphosate oxidoreductase plants displayed a superior glyphosate tolerance in that these plants did not show much, if any, immediate glyphosate effects at the treated rate. In general, the treated tolerant plants also showed normal development, flowering and fertility.

The CTP1-synthetic glyphosate oxidoreductase plants showed a markedly higher level of glyphosate oxidoreductase expression than that shown for the other glyphosate oxidoreductase constructs. This increased glyphosate oxidoreductase level could be due to enhancement of translation of the fusion or to sequestering of glyphosate oxidoreductase within the chloroplast and leading to a longer protein half-life. The higher level of glyphosate oxidoreductase and/or its location in the chloroplast can result in higher levels of glyphosate tolerance through rapid detoxification of glyphosate in the chloroplast. The presence of glyphosate oxidoreductase within the chloroplast has been confirmed. Five leaves from each of four plants (#22844, 22854, 22886, 22887), shown to be Western positive for glyphosate oxidoreductase, were homogenized in Waring blender in 0.9 L GR+ buffer (Bartlett, et al., 1982) for 3×3 seconds at high speed. The homogenate was filtered through 4 layers of Miracloth and centrifuged at 6,000 rpm in a GS-3 rotor. The pellet was resuspended in 4 ml total of GR+ buffer and placed on top of a 40/80% Percoll step gradient and spun at

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9,500 rpm for 10 minutes. The intact chloroplasts (lower band) were washed once with GR-buffer (Bartlett, et al., 1982) and centrifuged (up to 6,000 rpm with brake off). They were then resuspended in 300 μ l 50 mM Hepes pH 7.7, 330 mM Sorbitol and lysed on ice using by sonication (small probe, 30%—3 microtip setting \times 10 seconds). The debris was pelleted and the supernatant passed through a Sephadex G50 column into 50 mM Hepes, pH 7.5. The soluble protein concentration was 2.4 mg/ml. The enzyme assays were done as above using both 50 mM IDA and 50 mM glyphosate as substrates (30 minute assays), but without the addition of 1 mM ubiquinone.

TABLE IX

Glyphosate Oxidoreductase Activity in Isolated Chloroplast from Transgenic Tobacco		15
Substrate	Specific Activity (nmol/min mg)	
Iminodiacetic acid	179	20
Glyphosate	92	

Example 3

A number of transformed lines of canola have been derived with vectors pMON17138 (CTP1-synthetic glyphosate oxidoreductase) and pMON17164 (CTP2-synthetic glyphosate oxidoreductase) as follows.

Plant Material

Seedlings of *Brassica napus* cv Westar were established in 2 inch (~5 cm) pots containing Metro Mix 350. They were grown in a growth chamber at 24° C., 16/8 hour photoperiod, light intensity of 400 μ Em⁻²sec⁻¹ (HID lamps). They were fertilized with Peters 20-10-20 General Purpose Special. After 2½ weeks they were transplanted to 6 inch (~15 cm) pots and grown in a growth chamber at 15/10° C. day/night temperature, 16/8 hour photoperiod, light intensity of 800 μ Em⁻²sec⁻¹ (HID lamps). They were fertilized with Peters 15-30-15 Hi-Phos Special.

Transformation/Selection/Reigneneration

Four terminal internodes from plants just prior to bolting or in the process of bolting but before flowering were removed and surfaced sterilized in 70% v/v ethanol for 1 minute, 2% w/v sodium hypochlorite for 20 minutes and rinsed 3 times with sterile deionized water. Stems with leaves attached could be refrigerated in moist plastic bags for up to 72 hours prior to sterilization. Six to seven stem segments were cut into 5mm discs with a Redco Vegetable Slicer 200 maintaining orientation of basal end.

The Agrobacterium was grown overnight on a rotator at 24° C. in 2 mls of Luria Broth containing 50 mg/l kanamycin, 24 mg/l chloramphenicol and 100 mg/l spectinomycin. A 1:10 dilution was made in MS (Murashige and Skoog) media giving approximately 9×10^8 cells per ml. This was confirmed with optical density readings at 660 m μ . The stem discs (explants) were inoculated with 1.0 ml of Agrobacterium and the excess was aspirated from the explants.

The explants were placed basal side down in petri plates containing 1/10 \times standard MS salts, B5 vitamins, 3% sucrose, 0.8% agar, pH 5.7, 1.0 mg/l 6-benzyladenine (BA). The plates were layered with 1.5 ml of media containing MS salts, B5 vitamins, 3% sucrose, pH 5.7, 4.0 mg/l p-chlorophenoxyacetic acid, 0.005 mg/l kinetin and covered with sterile filter paper.

Following a 2 to 3 day co-culture, the explants were transferred to deep dish petri plates containing MS salts, B5

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vitamins, 3% sucrose, 0.8% agar, pH 5.7, 1 mg/l BA, 500 mg/l carbenicillin, 50 mg/l cefotaxime, 200 mg/l kanamycin or 175 mg/l gentamicin for selection. Seven explants were placed on each plate. After 3 weeks they were transferred to fresh media, 5 explants per plate. The explants were cultured in a growth room at 25° C., continuous light (Cool White).

Expression Assay

After 3 weeks shoots were excised from the explants. Leaf recallusing assays were initiated to confirm modification of R_0 shoots. Three tiny pieces of leaf tissue were placed on recallusing media containing MS salts, B5 vitamins, 3% sucrose, 0.8% agar, pH 5.7, 5.0 mg/l BA, 0.5 mg/l naphthalene acetic acid (NAA), 500 mg/l carbenicillin, 50 mg/l cefotaxime and 200 mg/l kanamycin or gentamicin or 0.5 mM glyphosate. The leaf assays were incubated in a growth room under the same conditions as explant culture. After 3 weeks the leaf recallusing assays were scored for herbicide tolerance (callus or green leaf tissue) or sensitivity (bleaching).

Transplantation

At the time of excision, the shoot stems were dipped in Rootone® and placed in 2 inch (~5 cm) pots containing Metro-Mix 350 and placed in a closed humid environment. They were placed in a growth chamber at 24° C., 16/8 hour photoperiod, 400 $\mu\text{Em}^{-2}\text{sec}^{-2}$ (HID lamps) for a hardening-off period of approximately 3 weeks.

The seed harvested from R_0 plants is R_1 seed which gives rise to R_1 plants. To evaluate the glyphosate tolerance of an R_0 plant, its progeny are evaluated. Because an R_0 plant is assumed to be hemizygous at each insert location, selfing results in maximum genotypic segregation in the R_1 . Because each insert acts as a dominant allele, in the absence of linkage and assuming only one hemizygous insert is required for tolerance expression, one insert would segregate 3:1, two inserts, 15:1, three inserts 63:1, etc. Therefore, relatively few R_1 plants need be grown to find at least one resistant phenotype.

Seed from an R_0 plant is harvested, threshed, and dried before planting in a glyphosate spray test. Various techniques have been used to grow the plants for R_1 spray evaluations. Tests are conducted in both greenhouses and growth chambers. Two planting systems are used; ~10 cm pots or plant trays containing 32 or 36 cells. Soil used for planting is either Metro 350 plus three types of slow release fertilizer or plant Metro 350. Irrigation is either overhead in greenhouses or subirrigation in growth chambers. Fertilizer is applied as required in irrigation water. Temperature regimes appropriate for canola were maintained. A sixteen hour photoperiod was maintained. At the onset of flowering, plants are transplanted to ~15 cm pots for seed production.

A spray "batch" consists of several sets of R_1 progenies all sprayed on the same date. Some batches may also include evaluations of other than R_1 plants. Each batch also includes sprayed and unsprayed non-transgenic genotypes representing the genotypes in the particular batch which were putatively transformed. Also included in a batch is one or more non-segregating transformed genotypes previously identified as having some resistance.

Two-six plants from each individual R_0 progeny are not sprayed and serve as controls to compare and measure the glyphosate tolerance, as well as to assess any variability not induced by the glyphosate. When the other plants reach the 2-4 leaf stage, usually 10 to 20 days after planting, glyphosate is applied at rates varying from 0.28 to 1.12 kg/ha, depending on objectives of the study. Low rate technology using low volumes has been adopted. A laboratory track sprayer has been calibrated to deliver a rate equivalent to field conditions.

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A scale of 0 to 10 is used to rate the sprayed plants for vegetative resistance. The scale is relative to the unsprayed plants from the same R_0 plant. A 0 is death, while a 10 represents no visible difference from the unsprayed plant. A higher number between 0 and 10 represents progressively less damage as compared to the unsprayed plant. Plants are scored at 7, 14, and 28 days after treatment (DAT), or until bolting, and a line is given the average score of the sprayed plants within an R_0 plant family.

Six integers are used to qualitatively describe the degree of reproductive damage from glyphosate:

- 0: No floral bud development
- 2: Floral buds present, but aborted prior to opening
- 4: Flowers open, but no anthers, or anthers fail to extrude past petals
- 6: Sterile anthers
- 8: Partially sterile anthers
- 10: Fully fertile flowers

Plants are scored using this scale at or shortly after initiation of flowering, depending on the rate of floral structure development.

Tables X and XI below tabulate the vegetative and reproductive scores for canola plants transformed with pMON17138 (sprayed at a rate of 0.56 kg/ha and pMON17164 (sprayed at a rate of 0.84 kg/ha), respectively. The results presented below illustrate the glyphosate tolerance conferred to canola plants as a result of expression of a glyphosate oxidoreductase gene in the plants.

TABLE X

Glyphosate Spray Evaluation of Canola Plants containing pMON17138			
Line name	Batch	0.56 kg/ha score 14 DAT	0.56 kg/ha score 28 DAT
		Vegetative	Reproductive
17138-22	79	9	10
17138-30	79	9	10
17138-145	79	10	10
17138-158	79	8	10
17138-164	80	8	10
Untransformed	77	3	0
Untransformed	79	1	0

TABLE XI

Glyphosate Spray Evaluation of Canola Plants containing pMON17164			
Construct	Batch	0.84 kg/ha score	
		14 DAT vegetative	28 DAT reproductive
17164-6	82	7	10
17164-9	83	8	10
17164-20	82	7	10
17164-25	83	8	10
17164-35	84	7	10
17164-45	83	9	10
17164-61	83	7	10
17164-75	84	7	10
17164-85	84	7	10
17164-97	84	6	10
17164-98	83	9	10
17164-105	83	7	10
17164-110	83	9	10
17164-115	83	7	10
17164-129	83	8	10

TABLE XI-continued

Glyphosate Spray Evaluation of Canola Plants containing pMON17164				
0.84 kg/ha score				
	Construct	Batch	14 DAT vegetative	28 DAT reproductive
5				
10	17164-139	84	7	10
	17164-140	83	8	10
	17164-164	83	7	10
	17164-166	83	8	10
	17164-174	83	8	10
	17164-186	83	3	10
15	17164-202	83	8	10
	17164-218	84	6	10
	17164-219	83	9	10
	17164-222	84	7	10
	17164-225	83	7	10
	17164-227	84	7	10
20	17164-230	83	8	10
	17164-243	83	7	10
	17164-247	84	7	10
	17164-287	84	7	10
	17164-289	83	8	10
	17164-300	83	9	10
25	17164-337	83	8	10

Example 4

The glyphosate oxidoreductase gene has also been introduced into and expressed in soybean and imparts glyphosate tolerance to such plants. The CTP2-synthetic glyphosate oxidoreductase fusion gene (as described above) was introduced into soybean under the control of the FMV promoter and with the NOS 3' sequences in vector pMON17159, a map of which is presented in FIG. 10. This vector consists of the following elements in addition to the glyphosate oxidoreductase gene sequences; the pUC origin of replication, an NPTII bacterial selectable marker gene (kanamycin) and the beta-glucuronidase gene (GUS; Jefferson et al. 1986) under the control of the E35S promoter and with the E9 3' sequences. The latter gene provides a scorable marker to facilitate the identification of transformed plant material.

Soybean plants are transformed with pMON17159 by the method of microprojectile injection using particle gun technology as described in Christou et al. (1988). The seed harvested from R_0 plants is R_1 seed which gives rise to R_1 plants. To evaluate the glyphosate tolerance of an R_0 plant, its progeny are evaluated. Because an R_0 plant is assumed to be hemizygous at each insert location, selfing results in maximum genotypic segregation in the R_1 . Because each insert acts as a dominant allele, in the absence of linkage and assuming only one hemizygous insert is required for tolerance expression, one insert would segregate 3:1, two inserts, 15:1, three inserts 63:1, etc. Therefore, relatively few R_1 plants need be grown to find at least one resistant phenotype.

Seed from an R_0 soybean plant is harvested, and dried before planting in a glyphosate spray test. Seeds are planted into 4 inch (~5cm) square pots containing Metro 350. Twenty seedlings from each R_0 plant is considered adequate for testing. Plants are maintained and grown in a greenhouse environment. A 12.5-14 hour photoperiod and temperatures of 30° C. day and 24° C. night is regulated. Water soluble Peters Lite fertilizer is applied as needed.

A spray "batch" consists of several sets of R_1 progenies all sprayed on the same date. Some batches may also include evaluations of other than R_1 plants. Each batch also includes

sprayed and unsprayed non-transgenic genotypes representing the genotypes in the particular batch which were putatively transformed. Also included in a batch is one or more non-segregating transformed genotypes previously identified as having some resistance.

One to two plants from each individual R_0 progeny are not sprayed and serve as controls to compare and measure the glyphosate tolerance, as well as to assess any variability not induced by the glyphosate. When the other plants reach the first trifoliolate leaf stage, usually 2–3 weeks after planting, glyphosate is applied at a rate equivalent of 128 oz./acre (8.895 kg/ha) of Roundup®. A laboratory track sprayer has been calibrated to deliver a rate equivalent to those conditions.

A vegetative score of 0 to 10 is used. The score is relative to the unsprayed progenies from the same R_0 plant. A 0 is death, while a 10 represents no visible difference from the unsprayed plant. A higher number between 0 and 10 represents progressively less damage as compared to the unsprayed plant. Plants are scored at 7, 14, and 28 days after treatment (DAT).

TABLE XII

Glyphosate Spray Evaluation of Soybean Plants containing pMON17159		
Line	Batch	Score @ 8 895 kg/ha. 28 DAT
17159-24	14	9
17159-25	14	9
17159-28	14	6
17159-40	14	4
17159-43	14	4
17159-71	14	10
17159-77	14	9
17159-81	15	4
Untransformed	14	0

Example 5

The glyphosate oxidoreductase gene has also been introduced into Black Mexican Sweet (BMS) corn cells with expression of the protein detected in callus.

Plasmid pMON19632 was used to introduce the glyphosate oxidoreductase gene into corn cells. The backbone for this plasmid was constructed by inserting the 0.6 kb cauliflower mosaic virus (CaMV) 35S RNA promoter (E35S) containing a duplication of the -90 to -300 region (Kay et al., 1987), a 0.58 kb fragment containing the first intron from the maize alcohol dehydrogenase gene (Callis et al., 1987), and the 3' termination sequences from the nopaline synthase (NOS) gene (Fraley et al., 1983) into pUC119 (Yanisch-Perron et al., 1985). pMON19632 was formed by inserting the 1.7 kb BglIII/EcoRI fragment from pMON17064 which contains the Arabidopsis SSU CTP fused to the synthetic glyphosate oxidoreductase coding sequence (SEQ IN NO:8).

Plasmid pMON19632 was introduced into BMS corn cells by co-bombardment with EC9, a plasmid containing a sulfonylurea-resistant form of the maize acetolactate synthase gene. 2.5 -g of each plasmid was coated onto tungsten particles and introduced into log-phase BMS cells using a PDS-1000 particle gun essentially as described in Klein et al., 1989. Transformants were selected on MS medium containing 20ppb chlorsulfuron. After initial selection on chlorsulfuron, the calli was assayed by glyphosate oxidoreductase Western blot.

BMS callus (3 g wet weight) was dried on filter paper (Whatman#1) under vacuum, reweighed, and extraction

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buffer (500 lg dry weight; 100 mM Tris, 1 mM EDTA, 10% glycerol) was added. The tissue was homogenized with a Wheaton overhead stirrer for 30 seconds at 2.8 power setting. After centrifugation (3 minutes, Eppendorf microfuge), the supernatant was removed and the protein was quantitated (BioRad Protein Assay). Samples (50 g/well) were loaded on an SDS PAGE gel (Jule, 3-17%) along with glyphosate oxidoreductase standard (10 ng), electrophoresed, and transferred to nitrocellulose similarly to a previously described method (Padgett, 1987). The nitrocellulose blot was probed with goat anti-glyphosate oxidoreductase IgG, and developed with I-125 Protein G. The radioactive blot was visualized by autoradiography. Results were quantitated by densitometry on an LKB UltraScan XL laser densitomer and are tabulated below in Table XIII.

TABLE XIII

Expression of glyphosate oxidoreductase in BMS Corn Callus using pMON19632		
Line	GOX expression (% extracted protein)	
EC9 (no GOX)	0	
T13-17	0.016	
T13-16	0.0065	
T13-15	0.016	
T13-14	0.003	
T13-12	0.0079	
T13-7	0.01	
T13-5	0.004	
T13-18	0.026	
T13-8	0.019	
T13-9	0.01	
T13-4	0.027	

Table XIII illustrates that glyphosate oxidoreductase can be expressed and detected in a monocotyledonous plant, such as corn.

Example 6

The glyphosate oxidoreductase gene may be used as a selectable marker for plant transformation directly on media containing glyphosate. The ability to select and to identify transformed plant material depends, in most cases, on the use of a dominant selectable marker gene to enable the preferential and continued growth of the transformed tissues in the presence of a normally inhibitory substance. Antibiotic resistance and herbicide tolerance genes have been used almost exclusively as such dominant selectable marker genes in the presence of the corresponding antibiotic or herbicide. The nptII/kanamycin selection scheme is probably the most frequently used. It has been demonstrated that glyphosate oxidoreductase is also a useful and perhaps superior selectable marker/selection scheme for producing and identifying transformed plants.

A plant transformation vector that may be used in this scheme is pMON17226 (FIG. 11). This plasmid resembles many of the other plasmids described infra and is essentially composed of the previously described bacterial replicon system that enables this plasmid to replicate in *E. coli* and to be introduced into and to replicate in *Agrobacterium*, the bacterial selectable marker gene (Spc/Str), and located between the T-DNA right border and left border is the CTP1-glyphosate oxidoreductase synthetic gene in the FMV promoter-E9 3' cassette. This plasmid also has single sites for a number of restriction enzymes, located within the borders and outside of the expression cassette. This makes it

possible to easily add other genes and genetic elements to the vector for introduction into plants.

The protocol for direct selection of transformed plants on glyphosate is outlined for tobacco. Explants are prepared for pre-culture as in the standard procedure as described in Example 1: surface sterilization of leaves from 1 month old tobacco plants (15 minutes in 10% clorox +surfactant; 3x dH₂O washes); explants are cut in 0.5x0.5 cm squares, removing leaf edges, mid-rib, tip, and petiole end for uniform tissue type; explants are placed in single layer, upside down, on MS104 plates+2 ml 4COO5K media to moisten surface; pre-culture 1-2 days. Explants are inoculated using overnight culture of *Agrobacterium* containing the plant transformation plasmid that is adjusted to a titer of 1.2×10^9 bacteria/ml with 4COO5K media. Explants are placed into a centrifuge tube, the *Agrobacterium* suspension is added and the mixture of bacteria and explants is "Vortexed" on maximum setting for 25 seconds to ensure even penetration of bacteria. The bacteria are poured off and the explants are blotted between layers of dry sterile filter paper to remove excess bacteria. The blotted explants are placed upside down on MS104 plates+2 ml 4COO5K media +filter disc. Co-culture is 2-3 days. The explants are transferred to MS104 +Carbenicillin 1000 mg/l +cefotaxime 100 mg/l for 3 days (delayed phase). The explants are then transferred to MS104 +glyphosate 0.05 mM +Carbenicillin 1000 mg/l+cefotaxime 100 mg/l for selection phase. At 4-6 weeks shoots are cut from callus and placed on MSO +Carbenicillin 500 mg/l rooting media. Roots form in 3-5 days, at which time leaf pieces can be taken from rooted plates to confirm glyphosate tolerance and that the material is transformed.

The presence of the glyphosate oxidoreductase protein in these transformed tissues has been confirmed by immunoblot analysis of leaf discs. The data from one experiment with pMON17226 is presented in the following: 25 shoots formed on glyphosate from 100 explants inoculated with *Agrobacterium* ABI/pMON17226; 15 of these were positive on reculturing on glyphosate, and 19 of these were positive for glyphosate oxidoreductase protein as detected by immunoblot.

These data indicate a transformation rate of 15-19 per 100 explants, which makes this a highly efficient and time saving transformation procedure for plant. Similar transformation frequencies have been obtained with a pMON17226 derivative (pMON17241) containing the gene for the glyphosate oxidoreductase v.247 (SEQ ID NO:17). The glyphosate oxidoreductase gene has also been shown to enable direct selection of transformants in other plant species, including *Arabidopsis*, potato, and sugarbeet.

From the foregoing, it will be seen that this invention is one well adapted to attain all the ends and objects herein-above set forth together with advantages which are obvious and which are inherent to the invention.

It will be understood that certain features and subcombinations are of utility and may be employed without reference to other features and subcombinations. This is contemplated by and is within the scope of the claims.

Since many possible embodiments may be made of the invention without departing from the scope thereof, it is to be understood that all matter herein set forth or shown in the accompanying drawings is to be interpreted as illustrative and not in a limiting sense.

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ATTCCTCTAG TACAAGTGGG GAACAAAATA ACGTGGAAAA GAGCTGTCCT GACAGCCAC	480
TCACTAATGC GTATGACGAA CGCAGTGACG ACCACAAAAG AATTTTCCT CTATATAAGA	540
AGGCATTTC TTTCCATTG AAGG	564

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 27 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATCATCAGAT ACTAACCAAT ATTCTC

27

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 1692 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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CACCTACCGC GAAACGGTGG CTAGGCAGCG AGAGACTGTC GGCTCCGCGG GAGCATCCTA	120
TGTCTGAGAA CCACAAAAAA GTAGGCATCG CTGGAGCCGG AATCGTCGGC GTATGCACGG	180
CGCTGATGCT TCAGCGCCGC GGATTCAAA G TCACCTTGAT TGACCCGAAC CCTCCTGGCG	240
AAGGTGCATC GTTTGGGAAT GCCGGATGCT TCAACGGCTC ATCCGTCGTC CCTATGTCCA	300
TGCCGGGAAA CTTGACGAGC GTGCCGAAGT GGCTCCTTGA CCCGATGGGG CCGTTGTCAA	360
TCCGGTTTCA CTATTTTCCA ACCATCATGC CCTGGTTGAT TCGCTTTCTG TTAGCCGGAA	420
GACCAAAACA GGTGAAGGAG CAGGCGAAA G CACTCCGCAA TCTCATCAAG TCCACGGTGC	480
CTCTGATCAA GTCATTTGGC GAGGAAGCTG ATGCGAGCCA TCTGATCCGC CATGAAGGTC	540
ATCTGACCGT ATATCGTGA GAAGCAGACT TCGCCAAGGA CCGCGGAGGT TGGGAACTGC	600
GGCGTCTCAA CGGTGTTTCG ACOCAGATCC TCAGCGCCGA TGCGTTGCGG GATTTTCGATC	660
CGAACTTGTC GCATGCGTTT ACCAAGGGCA TTCTTATAGA AGAGAACGGT CACACGATTA	720
ATCCGCAAGG GCTCGTGACC CTCTTGTTTC GCGCTTTTAT CGCGAACGGT GCGGAATTCT	780
TATCTGCGCG TGTATCGGC TTTGAGACTG AAGGTAGGGC GCTTAAAGGC ATTACAACCA	840
CGAACGGCGT TCTGGCCGTT GATOCAGCGG TTGTCGCAAC CGGCGCACAC TCGAAATCAC	900
TTGCTAATTC GCTAGGCGAT GACATCCCGC TCGATACCGA ACGTGGATAT CATATCGTCA	960
TCGCGAATCC GGAAGCCGCT CCACGCATT C GACGACCGA TGCGTCAGGA AAATTCATCG	1020
CGACACCTAT GGAAATGGGG CTTCGCGTGG CCGGTACGGT TGAGTTTCGT GGGCTCACAG	1080
CCGCTCCTAA CTGGAACGT GCGCATGTGC TCTATACGCA CGCTCGAAAA CTTCCTCCAG	1140
CCCTCGCGCC TCGGAGTTCT GAAGAACGAT ATTCCAAATG GATGGGGTTC CGGCCGAGCA	1200
TCCCGGATT C GCTCCCGTG ATTGGCCGGG CAACCCGGAC ACCCGACGTA ATCTATGCTT	1260
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195					200					205						
TTT	CGG	CGT	TTT	ATC	GCG	AAC	GGT	GGC	GAA	TTC	GTA	TCT	GCG	CGT	GTC	672
Phe	Arg	Arg	Phe	Ile	Ala	Asn	Gly	Gly	Glu	Phe	Val	Ser	Ala	Arg	Val	
	210					215					220					
ATC	GGC	TTT	GAG	ACT	GAA	GGT	AGG	GCG	CTT	AAA	GGC	ATT	ACA	ACC	ACG	720
Ile	Gly	Phe	Glu	Thr	Glu	Gly	Arg	Ala	Leu	Lys	Gly	Ile	Thr	Thr	Thr	
225					230					235					240	
AAC	GGC	GTT	CTG	GCC	GTT	GAT	GCA	GCG	GTT	GTC	GCA	GCC	GGC	GCA	CAC	768
Asn	Gly	Val	Leu	Ala	Val	Asp	Ala	Ala	Val	Val	Ala	Ala	Gly	Ala	His	
				245					250					255		
TCG	AAA	TCA	CTT	GCT	AAT	TCG	CTA	GGC	GAT	GAC	ATC	CCG	CTC	GAT	ACC	816
Ser	Lys	Ser	Leu	Ala	Asn	Ser	Leu	Gly	Asp	Asp	Ile	Pro	Leu	Asp	Thr	
			260					265					270			
GAA	CGT	GGA	TAT	CAT	ATC	GTC	ATC	GCG	AAT	CCG	GAA	GCC	GCT	CCA	CGC	864
Glu	Arg	Gly	Tyr	His	Ile	Val	Ile	Ala	Asn	Pro	Glu	Ala	Ala	Pro	Arg	
		275					280					285				
ATT	CCG	ACG	ACC	GAT	GCG	TCA	GGA	AAA	TTC	ATC	GCG	ACA	CCT	ATG	GAA	912
Ile	Pro	Thr	Thr	Asp	Ala	Ser	Gly	Lys	Phe	Ile	Ala	Thr	Pro	Met	Glu	
	290					295					300					
ATG	GGG	CTT	CGC	GTG	GCG	GGT	ACG	GTT	GAG	TTC	GCT	GGG	CTC	ACA	GCC	960
Met	Gly	Leu	Arg	Val	Ala	Gly	Thr	Val	Glu	Phe	Ala	Gly	Leu	Thr	Ala	
305					310					315					320	
GCT	CCT	AAC	TGG	AAA	CGT	GCG	CAT	GTG	CTC	TAT	ACG	CAC	GCT	CGA	AAA	1008
Ala	Pro	Asn	Trp	Lys	Arg	Ala	His	Val	Leu	Tyr	Thr	His	Ala	Arg	Lys	
				325					330				335			
CTT	CTT	CCA	GCC	CTC	GCG	CCT	GCG	AGT	TCT	GAA	GAA	CGA	TAT	TCC	AAA	1056
Leu	Leu	Pro	Ala	Leu	Ala	Pro	Ala	Ser	Ser	Glu	Glu	Arg	Tyr	Ser	Lys	
			340					345					350			
TGG	ATG	GGG	TTC	CGG	CCG	AGC	ATC	CCG	GAT	TCG	CTC	CCC	GTG	ATT	GGC	1104
Trp	Met	Gly	Phe	Arg	Pro	Ser	Ile	Pro	Asp	Ser	Leu	Pro	Val	Ile	Gly	
		355					360					365				
CGG	GCA	ACC	CGG	ACA	CCC	GAC	GTA	ATC	TAT	GCT	TTC	GGC	CAT	GGT	CAT	1152
Arg	Ala	Thr	Arg	Thr	Pro	Asp	Val	Ile	Tyr	Ala	Phe	Gly	His	Gly	His	
	370					375					380					
CTC	GGC	ATG	ACA	GGG	GCG	CCG	ATG	ACC	GCA	ACG	CTC	GTC	TCA	GAG	CTC	1200
Leu	Gly	Met	Thr	Gly	Ala	Pro	Met	Thr	Ala	Thr	Leu	Val	Ser	Glu	Leu	
	385				390					395					400	
CTC	GCA	GGC	GAA	AAG	ACC	TCA	ATC	GAC	ATT	TCG	CCC	TTC	GCA	CCA	AAC	1248
Leu	Ala	Gly	Glu	Lys	Thr	Ser	Ile	Asp	Ile	Ser	Pro	Phe	Ala	Pro	Asn	
				405					410					415		
CGC	TTT	GGT	ATT	GGC	AAA	TCC	AAG	CAA	ACG	GGT	CCG	GCA	AGT	TAA		1293
Arg	Phe	Gly	Ile	Gly	Lys	Ser	Lys	Gln	Thr	Gly	Pro	Ala	Ser			
			420					425					430			

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 430 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met Ser Glu Asn His Lys Lys Val Gly Ile Ala Gly Ala Gly Ile Val
1 5 10 15

Gly Val Cys Thr Ala Leu Met Leu Gln Arg Arg Gly Phe Lys Val Thr
20 25 30

Leu Ile Asp Pro Asn Pro Pro Gly Glu Gly Ala Ser Phe Gly Asn Ala
35 40 45

Gly Cys Phe Asn Gly Ser Ser Val Val Pro Met Ser Met Pro Gly Asn

5.776.760

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50					55					60					
Leu 65	Thr	Ser	Val	Pro	Lys 70	Trp	Leu	Leu	Asp	Pro 75	Met	Gly	Arg	Cys	Gln 80
Ser	Gly	Ser	Ala	Ile 85	Ser	Asn	His	His	Ala 90	Trp	Leu	Ile	Arg	Phe 95	Leu
Leu	Ala	Gly	Arg 100	Pro	Asn	Lys	Val	Lys 105	Glu	Gln	Ala	Lys	Ala 110	Leu	Arg
Asa	Leu	Ile 115	Lys	Ser	Thr	Val 120	Pro	Leu	Ile	Lys	Ser	Leu 125	Ala	Glu	Glu
Ala	Asp 130	Ala	Ser	His	Leu	Ile 135	Arg	His	Glu	Gly	His 140	Leu	Thr	Val	Tyr
Arg 145	Gly	Glu	Ala	Asp	Phe 150	Ala	Lys	Asp	Arg	Gly 155	Gly	Trp	Glu	Leu	Arg 160
Arg	Leu	Asn	Gly	Val 165	Arg	Thr	Gln	Ile	Leu 170	Ser	Ala	Asp	Ala	Leu 175	Arg
Asp	Phe	Asp	Pro 180	Asn	Leu	Ser	His	Ala 185	Phe	Thr	Lys	Gly	Ile 190	Leu	Ile
Glu	Glu	Asn 195	Gly	His	Thr	Ile	Asn 200	Pro	Gln	Gly	Leu	Val 205	Thr	Leu	Leu
Phe	Arg 210	Arg	Phe	Ile	Ala	Asn 215	Gly	Gly	Glu	Phe	Val 220	Ser	Ala	Arg	Val
Ile 225	Gly	Phe	Glu	Thr	Glu 230	Gly	Arg	Ala	Leu	Lys 235	Gly	Ile	Thr	Thr	Thr 240
Asn	Gly	Val	Leu	Ala 245	Val	Asp	Ala	Ala	Val 250	Val	Ala	Ala	Gly	Ala 255	His
Ser	Lys	Ser	Leu 260	Ala	Asn	Ser	Leu	Gly 265	Asp	Asp	Ile	Pro	Leu 270	Asp	Thr
Glu	Arg	Gly 275	Tyr	His	Ile	Val	Ile 280	Ala	Asn	Pro	Glu	Ala 285	Ala	Pro	Arg
Ile	Pro 290	Thr	Thr	Asp	Ala	Ser 295	Gly	Lys	Phe	Ile	Ala 300	Thr	Pro	Met	Glu
Met 305	Gly	Leu	Arg	Val	Ala 310	Gly	Thr	Val	Glu	Phe 315	Ala	Gly	Leu	Thr	Ala 320
Ala	Pro	Asn	Trp	Lys 325	Arg	Ala	His	Val	Leu 330	Tyr	Thr	His	Ala	Arg 335	Lys
Leu	Leu	Pro	Ala 340	Leu	Ala	Pro	Ala	Ser 345	Ser	Glu	Glu	Arg	Tyr 350	Ser	Lys
Trp	Met	Gly 355	Phe	Arg	Pro	Ser	Ile 360	Pro	Asp	Ser	Leu	Pro 365	Val	Ile	Gly
Arg	Ala 370	Thr	Arg	Thr	Pro	Asp 375	Val	Ile	Tyr	Ala	Phe 380	Gly	His	Gly	His
Leu 385	Gly	Met	Thr	Gly	Ala 390	Pro	Met	Thr	Ala	Thr 395	Leu	Val	Ser	Glu	Leu 400
Leu	Ala	Gly	Glu	Lys 405	Thr	Ser	Ile	Asp	Ile 410	Ser	Pro	Phe	Ala	Pro 415	Asn
Arg	Phe	Gly	Ile 420	Gly	Lys	Ser	Lys	Gln 425	Thr	Gly	Pro	Ala	Ser 430		

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1296 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

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AATCCGCAAG	GGCTCGTGAC	CCTCTTGTTT	CGGCGTTTTA	TCGCGAACGG	TGGCGAATTT	660
GTATCTGCGC	GTGTCATCGG	TTTTGAGACT	GAAGGTCGTG	CTCTCAAAGG	CATTACAACC	720
ACTAACGGTG	TTCTGGCTGT	TGATGCAGCT	GTGTTTGAG	CTGGTGCACA	CTCTAAATCA	780
CTTGCTAATT	CGCTAGGCGA	TGACATCCCG	CTCGATACCG	AACGTGGATA	TCATATCGTC	840
ATCGCGAATC	CGGAAGCCGC	TCCACGCATT	CCGACGACCG	ATGCGTCAGG	AAAATTCATC	900
GCGACACCTA	TGGAAATGGG	TCTTCGTGTT	GCTGGTACTG	TTGAGTTTGC	TGGTCTCACA	960
GCTGCTCCTA	ACTGGAACCG	TGCGCATGTG	CTCTATACGC	ACGCTCGAAA	ACTTCTTCCA	1020
GCCCTCGCGC	CTGCGAGTTC	TGAAGAACGA	TATTCCAAAT	GGATGGGTTT	TCGTCCTAGC	1080
ATTCTGATT	CTCTTCCAGT	GATTGGTCGT	GCAACTCGTA	CACCCGACGT	AATCTATGCT	1140
TTTGGTCACG	GTCTATCTCG	TATGACAGGT	GCTCCAATGA	CTGCAACTCT	CGTCTCAGAG	1200
CTCCTCGCAG	GCGAAAAGAC	CTCAATCGAC	ATTTGCCCCC	TCGCACCAAA	CCGCTTTGGT	1260
ATTGGCAAAAT	CCAAGCAAAC	GGGTCCGGCA	AGTTAA			1296

(2) INFORMATION FOR SEQ ID NO:8:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1296 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(1) MOLECULE TYPE: DNA (synthetic)

(1) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ATGGCTGAGA	ACCACAAGAA	GGTTGGTATC	GCTGGAGCTG	GAATCGTTGG	TGTTTGCAC	60
GCTTTGATGC	TTCAACGTCG	TGGATTCAAG	GTTACCTTGA	TTGATCCAAA	CCCACCAAGT	120
GAAGGTGCTT	CTTTCGGTAA	CGCTGGTTGC	TTCAACGGTT	CTTCCGTTGT	TCCAATGTCC	180
ATGCCAGGAA	ACTTGACTAG	CGTTCCAAAAG	TGGCTTCTTG	ACCCAATGGG	TCCATTGTCC	240
ATCCGTTTCA	GCTACTTTC	AACCATCATG	CCTTGGTTGA	TTGTTTCTT	GCTTGCTGGA	300
AGACCAAAACA	AGGTGAAOGA	GCAAGCTAAG	GCACTCCGTA	ACCTCATCAA	GTCCACTGTG	360
CCTTTGATCA	AGTCCTTGGC	TGAGGAGGCT	GATGCTAGCC	ACCTTATCCG	TCACGAAGGT	420
CACCTTACCG	TGTACCGTGG	AGAAGCAGAC	TTCGCCAAGG	ACCGTGGAGG	TTGGGAACIT	480
CGTCGTCTCA	ACGGTGTTCC	TACTCAAATC	CTCAGCGCTG	ATGCATTGCG	TGATTTGAT	540
CCTAACTTGT	CTCAGCCCTT	TACCAAGGGA	ATCCTTATCG	AAGAGAACGG	TCACACCATC	600
AACCCACAAG	GTCTCGTGAC	TCTCTTGTTT	CGTCGTTTCA	TCGCTAACGG	TGGAGAGTTC	660
GTGTCTGCTC	GTGTTATCGG	ATTCGAGACT	GAAGGTCGTG	CTCTCAAGGG	TATCACCACC	720
ACCAACGGTG	TTCTTGCTGT	TGATGCAGCT	GTGTTTGAG	CTGGTGCACA	CTCCAAGTCT	780
CTTGCTAACT	CCCTTGGTGA	TGACATCCCA	TTGGATACCG	AACGTGGATA	CCACATCGTG	840
ATCGCCAACC	CAGAAGCTGC	TCCACGTATT	CCAACCTACG	ATGCTTCTGG	AAAGTTCATC	900
GCTACTCCTA	TGGAGATGGG	TCTTCGTGTT	GCTGGAACCG	TTGAGTTCGC	TGGTCTCACT	960
GCTGCTCCTA	ACTGGAAGCG	TGCTCACGTT	CTCTACACTC	ACGCTCGTAA	GTTGCTTCCA	1020
GCTCTCGCTC	CTGCCAGTTC	TGAAGAACGT	TACTCCAAGT	GGATGGGTTT	CCGTCCAAGC	1080
ATCCAGATT	CCCTTCCAGT	GATTGGTCGT	GCTACCCGTA	CTCCAGACGT	TATCTACGCT	1140
TTGCGTCACG	GTACCTCGG	TATGACTGGT	GCTCCAATGA	CCGCAACCCT	CGTTTCTGAG	1200
CTCCTCGCAG	GTGAGAAGAC	CTCTATCGAC	ATCTCTCCAT	TCGCACCAAA	CCGTTTCTGGT	1260

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ATTGGTAAAGT CCAAGCAAAC TGGTCCTGCA TCCTAA

1296

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 279 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (recombinant)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
AGATCICAC AATGGCTTCC TCTATGCTCT CTTCCGCTAC TATGGTTGCC TCTCCGGCTC      60
AGGCCACTAT GGTGCTCCTT TCAACGGAC TTAAGTCCTC CGCTGCCTTC CCAGCCACCC      120
GCAAGGCTAA CAACGACATT ACTTCCATCA CAAGCAACGG CGGAAGAGTT AACTGCATGC      180
AGGTGTGGCC TCCGATTGGA AAGAAGAAAGT TTGAGACTCT CTCITACCTT CCTGACCTTA      240
CCGATTCCGG TGGTCGCGTC AACTGCATGC AGGCCATGG      279
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 318 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (recombinant)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
AGATCTATCG ATAAGCTTGA TGTAATTGGA GGAAGATCAA AATTTTCAAT CCCCATTCTT      60
CGATTGCTTC AATTGAAGTT TCTCCGATGG CGCAAGTTAG CAGAATCTGC AATGGTGTGC      120
AGAACCCATC TCTTATCTCC AATCTCTCGA AATCCAGTCA ACGCAAATCT CCCTTATCGG      180
TTTCTCTGAA GACGCAGCAG CATCCACGAG CTTATCCGAT TTCGTCGTCG TGGGGATTGA      240
AGAAGAGTGG GATGACGTTA ATTGGCTCTG AGCTTCGTCC TCTTAAGGTC ATGTCTTCTG      300
TTTCCACGGC GTGCATGC      318
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 119 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
NCATGGACGT CTGATCGAAA TCGTCGTTAC CGCAGCAAGG TAAAGGCACGC CGAATTTTAT      60
CACCTACCGC GAAACGGTGG CTAGGCAGCG AGAGACTGTC GGCTCCGCGG GAGCATCCT      119
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 277 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:12:

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GTACTTACGC	GGTCGTGAGT	ACAGCGCAGA	GCCGGTGTCA	AGATCAATCT	GCACCTCGCA	60
ATCACCTCGG	AGACGCGAAA	TGGCGCAAAT	AGAACACATA	TTAACGAGTC	ACGCCCCGAA	120
GCCTTTGGGT	CACTACAGTC	AGGCGGCCCG	AGCGGGTGGA	TTCATTTCATG	TTTCCGGTCA	180
GCTTCCGATC	AAACCAGAAG	GCCAGTCGGA	GCAATCTGAC	GATCTCGTCG	ATAACCAGGC	240
CAGTCTCGTT	CTCCGGAATT	TGCTGGCCGT	ACTCGAG			277

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (synthetic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GAGAGACTGT	CGACTCCGCG	GGAGCATCAT	ATG	33
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(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (synthetic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GAACGAATCC	AAGCTTCTCA	CGACCGCGTA	AGTAC	35
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(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (synthetic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GCCGAGATGA	CCGTGGCCGA	AAGC	24
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(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (synthetic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GCGAATGCCG	GATGCTTCAA	CGGC	24
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(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1296 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (recombinant)

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(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: 1..1296

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

ATG	GCT	GAG	AAC	CAC	AAG	AAO	GTT	GGT	ATC	GCT	GGA	GCT	GGA	ATC	GTT	48
Met	Ala	Glu	Asn	His	Lys	Lys	Val	Gly	Ile	Ala	Gly	Ala	Gly	Ile	Val	
1				5					10					15		
GGT	GTT	TGC	ACT	GCT	TTG	ATG	CTT	CAA	CGT	CGT	GGA	TTC	AAG	GTT	ACC	96
Gly	Val	Cys	Thr	Ala	Leu	Met	Leu	Gln	Arg	Arg	Gly	Phe	Lys	Val	Thr	
			20					25					30			
TTG	ATT	GAT	CCA	AAC	CCA	CCA	GGT	GAA	GGT	GCC	TCT	TTC	GGT	AAC	GCT	144
Leu	Ile	Asp	Pro	Asn	Pro	Pro	Gly	Glu	Gly	Ala	Ser	Phe	Gly	Asn	Ala	
		35					40					45				
GGT	TGC	TTC	AAC	GGT	TCC	TCC	GTT	GTT	CCA	ATG	TCC	ATG	CCA	GGA	AAC	192
Gly	Cys	Phe	Asn	Gly	Ser	Ser	Val	Val	Pro	Met	Ser	Met	Pro	Gly	Asn	
	50					55					60					
TTG	ACT	AGC	GTT	CCA	AAG	TGG	CTT	CTT	GAC	CCA	ATG	GGT	CCA	TTG	TCC	240
Leu	Thr	Ser	Val	Pro	Lys	Trp	Leu	Leu	Asp	Pro	Met	Gly	Pro	Leu	Ser	
	65				70				75						80	
ATC	CGT	TTC	GGC	TAC	TTT	CCA	ACC	ATC	ATG	CCT	TGG	TTG	ATT	CGT	TTC	288
Ile	Arg	Phe	Gly	Tyr	Phe	Pro	Thr	Ile	Met	Pro	Trp	Leu	Ile	Arg	Phe	
				85					90					95		
TTG	CTT	GCT	GGA	AGA	CCA	AAC	AAG	GTG	AAG	GAG	CAA	GCT	AAG	GCA	CTC	336
Leu	Leu	Ala	Gly	Arg	Pro	Asn	Lys	Val	Lys	Glu	Gln	Ala	Lys	Ala	Leu	
			100					105					110			
CGT	AAC	CTC	ATC	AAG	TCC	ACT	GTG	CCT	TTG	ATC	AAG	TCC	TTG	GCT	GAG	384
Arg	Asn	Leu	Ile	Lys	Ser	Thr	Val	Pro	Leu	Ile	Lys	Ser	Leu	Ala	Glu	
		115					120					125				
GAG	GCT	GAT	GCT	AGC	CAC	CTT	ATC	CGT	CAC	GAA	GGT	CAC	CTT	ACC	GTG	432
Glu	Ala	Asp	Ala	Ser	His	Leu	Ile	Arg	His	Glu	Gly	His	Leu	Thr	Val	
	130					135					140					
TAC	CGT	GGA	GAA	GCA	GAC	TTC	GCC	AGG	GAC	CGT	GGA	GGT	TGG	GAA	CTT	480
Tyr	Arg	Gly	Glu	Ala	Asp	Phe	Ala	Arg	Asp	Arg	Gly	Gly	Trp	Glu	Leu	
	145				150				155					160		
CGT	CGT	CTC	AAC	GGT	GTT	CGT	ACT	CAA	ATC	CTC	AGC	GCT	GAT	GCA	TTG	528
Arg	Arg	Leu	Asn	Gly	Val	Arg	Thr	Gln	Ile	Leu	Ser	Ala	Asp	Ala	Leu	
			165					170						175		
CGT	GAT	TTC	GAT	CCT	AAC	TTG	TCT	CAC	GCC	TTT	ACC	AAG	GGA	ATC	CTT	576
Arg	Asp	Phe	Asp	Pro	Asn	Leu	Ser	His	Ala	Phe	Thr	Lys	Gly	Ile	Leu	
			180					185					190			
ATC	GAA	GAG	AAC	GGT	CAC	ACC	ATC	AAC	CCA	CAA	GGT	CTC	GTG	ACT	CTC	624
Ile	Glu	Glu	Asn	Gly	His	Thr	Ile	Asn	Pro	Gln	Gly	Leu	Val	Thr	Leu	
		195					200					205				
TTG	TTT	CGT	CGT	TTC	ATC	GCT	AAC	GGT	GGA	GAG	TTC	GTG	TCT	GCT	CGT	672
Leu	Phe	Arg	Arg	Phe	Ile	Ala	Asn	Gly	Gly	Glu	Phe	Val	Ser	Ala	Arg	
	210					215					220					
GTT	ATC	GGA	TTC	GAG	ACT	GAA	GGT	CGT	GCT	CTC	AAG	GGT	ATC	ACC	ACC	720
Val	Ile	Gly	Phe	Glu	Thr	Glu	Gly	Arg	Ala	Leu	Lys	Gly	Ile	Thr	Thr	
	225				230				235					240		
ACC	AAC	GGT	GTT	CTT	GCT	GTT	GAT	GCA	GCT	GTT	GTT	GCA	GCT	GCT	GCA	768
Thr	Asn	Gly	Val	Leu	Ala	Val	Asp	Ala	Ala	Val	Val	Ala	Ala	Gly	Ala	
			245					250						255		
CAC	TCC	AAG	TCT	CTT	GCT	AAC	TCC	CTT	GGT	GAT	GAC	ATC	CCA	TTG	GAT	816
His	Ser	Lys	Ser	Leu	Ala	Asn	Ser	Leu	Gly	Asp	Asp	Ile	Pro	Leu	Asp	
			260					265					270			
ACC	GAA	CGT	GGA	TAC	CAC	ATC	GTG	ATC	GCC	AAC	CCA	GAA	GCT	GCT	CCA	864
Thr	Glu	Arg	Gly	Tyr	His	Ile	Val	Ile	Ala	Asn	Pro	Glu	Ala	Ala	Pro	
		275					280					285				
CGT	ATT	CCA	ACT	ACC	GAT	GCT	TCT	GGA	AAG	TTC	ATC	GCT	ACT	CCT	ATG	912

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Arg	Ile	Pro	Thr	Thr	Asp	Ala	Ser	Gly	Lys	Phe	Ile	Ala	Thr	Pro	Met	
290						295					300					
GAG	ATG	GGT	CTT	CGT	GTT	GCT	GGA	ACC	GTT	GAG	TTC	GCT	GGT	CTC	ACT	960
Glu	Met	Gly	Leu	Arg	Val	Ala	Gly	Thr	Val	Glu	Phe	Ala	Gly	Leu	Thr	
305					310					315					320	
GCT	GCT	CCT	AAC	TGG	AAG	CGT	GCT	CAC	GTT	CTC	TAC	ACT	CGC	GCT	CGT	1008
Ala	Ala	Pro	Asn	Trp	Lys	Arg	Ala	His	Val	Leu	Tyr	Thr	Arg	Ala	Arg	
				325					330					335		
AAG	TTC	CTT	CCA	GCT	CTC	GCT	CCT	GCC	AGT	TCT	GAA	GAA	CGT	TAC	TCC	1056
Lys	Leu	Leu	Pro	Ala	Leu	Ala	Pro	Ala	Ser	Ser	Glu	Glu	Arg	Tyr	Ser	
			340					345					350			
AAG	TGG	ATG	GGT	TTC	CGT	CCA	AGC	ATC	CCG	GAT	TCC	CTT	CCA	GTG	ATT	1104
Lys	Trp	Met	Gly	Phe	Arg	Pro	Ser	Ile	Pro	Asp	Ser	Leu	Pro	Val	Ile	
	355						360					365				
GGT	CGT	GCT	ACC	CGT	ACT	CCA	GAC	GTT	ATC	TAC	GCT	TTC	GGT	CAC	GGT	1152
Gly	Arg	Ala	Thr	Arg	Thr	Pro	Asp	Val	Ile	Tyr	Ala	Phe	Gly	His	Gly	
370						375					380					
CAC	CTC	GGT	ATG	ACT	GGT	GCT	CCA	ATG	ACC	GCA	ACC	CTC	GTT	TCT	GAG	1200
His	Leu	Gly	Met	Thr		Gly	Ala	Pro	Met	Thr	Ala	Thr	Leu	Val	Ser	
385					390						395				400	
CTC	CTC	GCA	GGT	GAG	AAG	ACC	TCT	ATC	GAC	ATC	TCT	CCA	TTC	GCA	CCA	1248
Leu	Leu	Ala	Gly	Glu	Lys	Thr	Ser	Ile	Asp	Ile	Ser	Pro	Phe	Ala	Pro	
				405					410					415		
AAC	CGT	TTC	GGT	ATT	GGT	AAG	TCC	AAG	CAA	ACT	GGT	CCT	GCA	TCC	TAA	1296
Asa	Arg	Phe	Gly	Ile	Gly	Lys	Ser	Lys	Gln	Thr	Gly	Pro	Ala	Ser		
			420					425					430			

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 431 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Met Ala Glu Asn His Lys Lys Val Gly Ile Ala Gly Ala Gly Ile Val
 1 5 10 15

Gly Val Cys Thr Ala Leu Met Leu Glu Arg Arg Gly Phe Lys Val Thr
 20 25 30

Leu Ile Asp Pro Asn Pro Pro Gly Glu Gly Ala Ser Phe Gly Asn Ala
 35 40 45

Gly Cys Phe Asn Gly Ser Ser Val Val Pro Met Ser Met Pro Gly Asn
 50 55 60

Leu Thr Ser Val Pro Lys Trp Leu Leu Asp Pro Met Gly Pro Leu Ser
 65 70 75 80

Ile Arg Phe Gly Tyr Phe Pro Thr Ile Met Pro Trp Leu Ile Arg Phe
 85 90 95

Leu Leu Ala Gly Arg Pro Asn Lys Val Lys Glu Gln Ala Lys Ala Leu
 100 105 110

Arg Asn Leu Ile Lys Ser Thr Val Pro Leu Ile Lys Ser Leu Ala Glu
 115 120 125

Glu Ala Asp Ala Ser His Leu Ile Arg His Glu Gly His Leu Thr Val
 130 135 140

Tyr Arg Gly Glu Ala Asp Phe Ala Arg Asp Arg Gly Gly Trp Glu Leu
 145 150 155 160

Arg Arg Leu Asn Gly Val Arg Thr Gln Ile Leu Ser Ala Asp Ala Leu
 165 170 175

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Arg	Asp	Phe	Asp	Pro	Asn	Leu	Ser	His	Ala	Phe	Thr	Lys	Gly	Ile	Leu
			180					185					190		
Ile	Glu	Glu	Asn	Gly	His	Thr	Ile	Asn	Pro	Gln	Gly	Leu	Val	Thr	Leu
		195					200					205			
Leu	Phe	Arg	Arg	Phe	Ile	Ala	Asn	Gly	Gly	Glu	Phe	Val	Ser	Ala	Arg
	210					215					220				
Val	Ile	Gly	Phe	Glu	Thr	Glu	Gly	Arg	Ala	Leu	Lys	Gly	Ile	Thr	Thr
225					230					235					240
Thr	Asn	Gly	Val	Leu	Ala	Val	Asp	Ala	Ala	Val	Val	Ala	Ala	Gly	Ala
				245					250					255	
His	Ser	Lys	Ser	Leu	Ala	Asn	Ser	Leu	Gly	Asp	Asp	Ile	Pro	Leu	Asp
			260					265					270		
Thr	Glu	Arg	Gly	Tyr	His	Ile	Val	Ile	Ala	Asn	Pro	Glu	Ala	Ala	Pro
		275					280					285			
Arg	Ile	Pro	Thr	Thr	Asp	Ala	Ser	Gly	Lys	Phe	Ile	Ala	Thr	Pro	Met
	290					295					300				
Glu	Met	Gly	Leu	Arg	Val	Ala	Gly	Thr	Val	Glu	Phe	Ala	Gly	Leu	Thr
305					310					315					320
Ala	Ala	Pro	Asn	Trp	Lys	Arg	Ala	His	Val	Leu	Tyr	Thr	Arg	Ala	Arg
				325					330					335	
Lys	Leu	Leu	Pro	Ala	Leu	Ala	Pro	Ala	Ser	Ser	Glu	Glu	Arg	Tyr	Ser
			340					345					350		
Lys	Trp	Met	Gly	Phe	Arg	Pro	Ser	Ile	Pro	Asp	Ser	Leu	Pro	Val	Ile
		355					360					365			
Gly	Arg	Ala	Thr	Arg	Thr	Pro	Asp	Val	Ile	Tyr	Ala	Phe	Gly	His	Gly
	370					375					380				
His	Leu	Gly	Met	Thr	Gly	Ala	Pro	Met	Thr	Ala	Thr	Leu	Val	Ser	Glu
385					390					395					400
Leu	Leu	Ala	Gly	Glu	Lys	Thr	Ser	Ile	Asp	Ile	Ser	Pro	Phe	Ala	Pro
				405					410					415	
Asn	Arg	Phe	Gly	Ile	Gly	Lys	Ser	Lys	Gln	Thr	Gly	Pro	Ala	Ser	
			420					425					430		

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 29 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (synthetic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CGTTCCTCTAC ACTCGTGCTC GTAAGTTGC

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(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 29 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (synthetic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CGTTCCTCTAC ACTAAGGCTC GTAAGTTGC

29

(2) INFORMATION FOR SEQ ID NO:21:

-continued

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 29 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (synthetic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CGTTCTCTAC ACTCAAGCTC GTAAAGTTGC

29

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 29 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (synthetic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CGTTCTCTAC ACTGCTGCTC GTAAAGTTGC

29

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 32 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (synthetic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CTCTACACTT GGGCTCGTAA GCTTCTTCCA GC

32

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 32 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (synthetic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CTCTACACTA TCGCTCGTAA GCTTCTTCCA GC

32

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 32 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (synthetic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CTCTACACTC TGGCTCGTAA GCTTCTTCCA GC

32

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 32 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single

-continued

(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (synthetic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CTCTACACTG AAGCTCGTAA GCTTCTTCCA GC

3 2

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 62 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (synthetic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CGCTGGAGCT GGAATCGTTG GTGTATGCAC TGCTTIGATG CTTCAACGTC GTGGATTCAA

6 0

AG

6 2

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 65 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (synthetic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GCAGATCCTC TCTGCTGATG CTTTGCCTGA TTTCGATCCT AACTTGTCTC ATGCTTTTAC

6 0

CAAGG

6 5

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (synthetic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:29:

GTCATCGGTT TTGAGACTGA AGGTCGTGCT CTCAAAGGCA T

4 1

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 69 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (synthetic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:30:

TACAACCACT AACGGTGTTC TGGCTGTTGA TGCAGCTGTT GTTGCAGCTG GTGCACACTC

6 0

TAAATCACT

6 9

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 61 base pairs
- (B) TYPE: nucleic acid

-continued

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (synthetic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GGAAATGGGT CTTCGTGTTG CTGGTACTGT TGAGTTTGCT GGTCTCACAG CTGCTCCTAA 60
C 61

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 68 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (synthetic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:32:

TGGATGGGTT TTCGTCCTAG CATTCTGAT TCTCTTCCAG TGATTGGTCG TGCAACTCGT 60
ACACCCGA 68

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 69 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (synthetic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:33:

CGTAATCTAT GCTTTTGGTC ACGGTCATCT CGGTATGACA GGTGCTCCAA TGA CTGCAAC 60
TCTCCTCTC 69

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71

We claim:

40

1. An isolated double-stranded DNA molecule which hybridizes to the DNA sequence of SEQ ID NO:3, wherein said DNA molecule encodes a glyphosate oxidoreductase enzyme.

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72

- ⁴⁰ 2. A recombinant bacterium containing the double-stranded DNA molecule of claim 1.

* * * * *

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18. A bacterium containing the DNA molecule of claim 17.
19. A method for selecting transformed plant cells comprising:
- (a) introducing a chimeric gene comprising SEQ ID NO:3, 4, 6, 7, 8 or 17 into plant cells;
 - (b) placing said plant cells on a plant growth media containing glyphosate; and
 - (c) selecting plant cells that exhibit growth on said glyphosate containing media.
20. A method for selecting transformed plant cells comprising:
- (a) introducing a chimeric gene comprising a DNA molecule encoding a glyphosate oxidoreductase enzyme into plant cells, wherein the DNA molecule is capable of hybridizing to SEQ ID NO:3, 4, 6, 7, 8 or 17;
 - (b) placing said plant cells on a plant growth media containing glyphosate; and
 - (c) selecting plant cells that exhibit growth on said glyphosate containing media.

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SspI

6358 TCATCAAAATATTTAGCAGCATTCAGATTGGTTCAA
TCAACAAGGTACGAGCCATATCACTTTATTCAAATTGG
TATCGCCAAAACCAAGGAAGAACTCCCATCCTCAAAGG
TTTGTAAGGAAGAAATTCTCAGTCCAAAGCCTCAACAAG
GTCAGGGTACAGAGTCTCCAAACCATTAGCCAAAAGCT
ACAGGAGATCAATGAAGAACTTTCAATCAAAGTAAACT
ACTGTTCCAGCACATGCATCATGGTCAGTAAGTTTCAG
AAAAGACATCCACCGAAGACTTAAAGTTAGTGGGCAT
CTTTGAAAGTAATCTTGTCAACATCGAGCAGCTGGCTT
GTGGGACCAGACAAAAAAGGAATGGTGCAGAAATTGTT
AGGCGCACCTACCAAAAGCATCTTTGCCCTTTATTGCAA
AAGATAAGCAGATTCCCTCTAGTACAAGTGGGGAACAA

FIGURE 1A

004040"4042F950

AATAACGTGGAAGAGAGCTGTCTGACAGCCCACTCAC
TAATGCGTATGACGAACGCAGTGACGACCAAAAGAA
TTTTCCCTCTATATAAGAAGGCATTTCATTTCCCATTTG
AAGGATCATCAGATACTAACCAATATTTCTC 6954
SspI

FIGURE 1B

1002020-4042F960

1 NCATGGACGTCGTGATCGAAATCGTCGTTACCGCAGCAAGGTAAGGCACGCCGAATTTTAT
61 CACCTACCGGAAACGGTGGCTAGGCAGCGAGAGACTGTCGGCTCCGGCGGAGCATCCCTA
M ("MET120")
121 TGTCTGAGAACCAAAAAGTAGGCATCGCTGGAGCCGGAATCGTCGGCGTATGCACGG
S E N H K K V G I A G A G I V G V C T A
181 CGCTGATGCTTCAGCGCCCGGATTCAAAGTCACCTTGATTGACCCGAACCCCTCCTGGCG
L M L Q R R G F K V T L I D P N P P G E
241 AAGGTGCATCGTTTGGGAATGCCGGATGCTTCAACGGCTCATCCGTCGTCCTATGTCCA
G A S F G N A G C F N G S S V V P M S M

FIGURE 2A

004040 " 4042F950

301
TGCCGGAAACTTGACGAGCGTGCCGAAGTGGCTCCTTGACCCGATGGGGCCGTTGTCAA
P G N L T S V P K W L L D P M G P L S I

361
TCCGGTTCAGCTATTTCCAACCATCATGCCCTGGTTGATTGCTTCTGTTAGCCGGAA
R F S Y F P T I M P W L I R F L L A G R

421
GACCAACAAGTGAAGGAGCAGCGAAGCACTCCGCAATCTCATCAAGTCCACGGTGC
P N K V K E Q A K A L R N L I K S T V P

481
CTCTGATCAAGTCATTGGCGGAGGAGGCTGATGCGAGCCATCTGATCCGCCATGAAGGTC
L I K S L A E E A D A S H L I R H E G H

541
ATCTGACCGTATATCGTGGAGAAGCAGACTTCGCCAAGGACCGGAGGTTGGGAACTGC
L T V Y R G E A D F A K D R G G W E L R

FIGURE 2B

601
GGCGTCTCAACGGTGTTCGCACGCAGATCCTCAGCGCCGATGCGTTGCGGGATTTCGATC
R L N G V R T Q I L S A D A L R D F D P

661
SphI
CGAACTTGTCGATGCGTTTACCAAGGCAATTCTTATAGAAGAACGGTCACACGATTA
N L S H A F T K G I L I E E N G H T I N

721
EcoRI
ATCCGCAAGGCTCGTGACCCCTCTTGTTCGGCGTTTATCGGAACGGTGCGGAATTCG
P Q G L V T L L F R R F I A N G G E F V

781
TATCTGCGGTGTCATCGGCTTTGAGACTGAAGGTAGGGCGCTTAAAGGCATTACAACCA
S A R V I G F E T E G R A L K G I T T T

FIGURE 2C

841
CGAACGGCGTTCTGTGGCCGTTGATGCAGCGGTTGTGCGAGCCGGCGCACACTCGAAATCAT
N G V L A V D A A V A A G A H S K S L

901
EcoRV
TTGCTAATTCGCTAGCGATGACATCCCGCTCGATACCGAACGTGGATATCATATCGTCA
A N S L G D D I P L D T E R G Y H I V I

961
TCGCGAATCCGGAAGCCGCTCCACGCAATTCGACGACCGATGCGTCAGGAAAATTCATCG
A N P E A A P R I P T T D A S G K F I A

1021
CGACACCTATGGAATGGGGCTTCGCGTGGCGGTACGGTTGAGTTCGCTGGGCTCACAG
T P M E M G L R V A G T V E F A G L T A

FIGURE 2D

1081
CCGCTCCTAACTGGAAACGTGCGCATGTGCTCTATACGCACGCTCGAAACTTCTTCCAG
A P N W K R A H V L Y T H A R K L L P A

1141
CCCTCGGCCTGCGAGTTCTGAAGAACGATATTCCAATGGATGGGGTTCCGGCCGAGCA
L A P A S S E E R Y S K W M G F R P S I

1201
TCCCGGATTGCTCCCGTGATTGGCCGGCAACCCGGACACCCGACGTAATCTATGCTT
P D S L P V I G R A T R T P D V I Y A F

1261
NcoI
TCGGCCATGGTCATCTCGGCATGACAGGGCGCCGATGACCGCAACGCTCGTCTCAGAGC
G H G H L G M T G A P M T A T L V S E L
SacI

FIGURE 2E

1321
 TCCTCGAGCGGAAAGACCTCAATCGACATTTGCGCCTTCGCACCAACCGCTTTGGTA
 L A G E K T S I D I S P F A P N R F G I

1381
 ScaI
 TTGGCAATCCAAGCAACGGTCCGGCAAGTTAAGTACTTACGCGGTCTGTAGTACAGC
 G K S K Q T G P A S *

1441
 GCAGAGCCGTGTCAAGATCAATCTGCACCTCGCAATCACCTCGGAGACGCGAAATGGCG

1501
 CAAATAGAACACATATTAAACGAGTCACGCCCCGAAAGCCTTTGGGTCACTACAGTCAGGCG

1561
 GCCCGAGCGGTGGATTCAATTCATGTTTCCGGTCAGCTTCCGATCAAACCCAGAAGGCCAG

FIGURE 2F

004040" 4042F560

1621

TCGGAGCAATCTGACGATCTCGTCGATAACCAGGCCAGTCTCGTTCTCCGGAATTGCTG

1681

XhoI

GCCGTACTCGAG

FIGURE 2G

1 fMet
AGATCTCCATGGCTGAGAACCAACAAAAGTAGGCATCGCTGGAGCCGGA
T
--
51
ATCGTCGGCGTATGCACGGCGCTGATGCTTCAGCGCGCGGATTCAAAGT
T T T T T A T T
101
CACCTTGATTGACCCGAACCCCTCCTGGCGAAGGTGCATCGTTTGGGAATG
151
CCGGATGCTTCAACGGCTCATCCGTGTCCTCCCTATGTCCATGCCGGGAAC

FIGURE 3A

002020" 4042F960

201
TTGACGAGCGTGCCGAAGTGGCTCCTTGACCCGATGGGGCCGTTGTCAAT

251
CCGGTTCAGCTATTTTCCAACCATCATGCCCCCTGGTTGATTCGCCTTCTGT

301
TAGCCGAAGACCAACAAGGTGAAGGAGCAGGCGAAAGCACTCCGCAAT

351
CTCATCAAGTCCACGGTGCCCTCTGATCAAGTCATTGGCGGAGGAGGCTGA

401
TGCGAGCCATCTGATCCGCCCATGAAGTCACTGACCGTATATCCTGGAG

FIGURE 3B

002020" 4042F560

451
AAGCAGACTTCGCCAAGGACCGCGGAGGTTGGAACTGCCGGCGTCTCAAC

501
GGTGTTCGCACGCAGATCCTCAGCGCCGATGCGTTGCGGGATTTCGATCC
TCT T T T

551
GAACTTGTCGATGCGTTTACCAAGGCAATCTTATAGAAGAGAACGGTC
T T

601
ACACGATTAAATCCGCAAGGGCTCGTGACCCCTCTTGTTCGGCGTTTATC

FIGURE 3C

002020-4042F560

651
GCGAACGGTGGCGAATTGTATCTGCGCGTGTCAATCGGCTTTGAGACTGA
T

701
AGGTAGGGCGCTTAAAGGCATTACAACCAAGGCGTTCTGGCCGTTG
C T T C T T T

751
ATGCAGCGGTTGTGCGAGCCGGCGCACACTCGAAATCACTTGCTAATTCG
T T T T T

801
CTAGGCGATGACATCCCGCTCGATACCGAACGTGGATATCATATCGTCAT

FIGURE 3D

DDO20"4042F960

851

.
CGCGAATCCGGAAGCCGCTCCACGCATTCCGACGACCGATGCCGTCAGGAA

901

.
AATTCAATCGCGACACCTATGGAATGGGGCTTCGCGTGGCGGTACGGTT
T T T T T

951

.
GAGTTCGCTGGGCTCACAGCCGCTCCTAACTGGAACGTGCCATGTGCT
T T T

1001

.
CTATACGCACGCTCGAAAACTTCTCCAGCCCTCGCGCCTGCGAGTTCTG

FIGURE 3E

1051
AAGAACGATATTCCAAATGGATGGGGTTCCGGCCGAGCATCCCGGATTCG
T T T T T T T
1101
CTCCCCGTGATTGGCCGGGCAACCCGGACACCCGACGTAATCTATGCTTT
T A T T T T T
1151
CGGCCACGGTCACTCGGCATGACAGGGGCGCCGATGACCGCAACGCTCG
T T T T A T T
1201
TCTCAGAGCTCCTCGCAGGCGAAAGACCCTCAATCGACATTCGCCCTTC

FIGURE 3F

004040" 4042F960

1251

GCACCAAACCGCTTTGGTATTGGCAAAATCCAAGCAACGGGTCCGGCAAG

1301

TTAAGTGGGAATTCAAGCTTG

FIGURE 3G

002020" 4042F950

1
AGATCTCCATGGCTGAGAACCAAAAAAGTAGGCATCGCTGGAGCCGGA
G G T T T

51
ATCGTCGGCGTATGCACGGCGCTGATGCTTCAGCGCCGCGATTCAAAGT
T T T T T A T T G

101
CACCTTGATTGACCCGAACCCCTCCTGGCGAAGGTGCATCGTTTGGGAATG
T A A A T T T C T C

151
CCGGATGCTTCAACGGCTCATCCGTCGTCCTATGTCCATGCCGGGAAC
T T T C T T A A

FIGURE 4A

002020" 4042F560

201

TTGACGAGCGTGCCGAAGTGGCTCCTTGACCCGATGGGGCCGTTGTCAAT
T T A T A T A C

251

CCGGTTCAGCTATTTCCAAACCATCATGCCCTGGTTGATTGCTTTCTGT
T C T T CT C

301

TAGCCGGAAGACCAACAAGGTGAAGGAGCAGGCGAAAGCACTCCGCAAT
T T A T G T C

351

CTCATCAAGTCCCGTGCCCTCTGATCAAGTCATTGGCGGAGGAGGCTGA
T T C T

FIGURE 4B

002020" 4042T960

401

· · · · ·
TGGAGCCATCTGATCCGCCATGAAGTCACTGACCGTATATCGTGGAG
T C T T C C T G C

451

· · · · ·
AAGCAGACTTCGCCAAGGACCGCGGAGGTTGGGAAC TGCGGCGTCTCAAC
T T T

501

· · · · ·
GGTGTTCGCACGCAGATCCTCAGCGCCGATGCGTTGCGGGA TTTCGATCC
T T A T A T

551

· · · · ·
GAACTTGTCGCATGCGTTTACCAAGGCGATCTTATAGAAGAACGGTC
T T C C A C C

FIGURE 4C

601
ACACGATTAAATCCGCAAGGCTCGTGACCCCTCTTGTTTCGGCGTTTATC
C C C A T T T C

651
GCCAACGGTGGCGAATTGTATCTGCGCGTGTATCGGCTTGAGACTGA
T A G C G T T A C

701
AGGTAGGGCGCTTAAAGGCATTACAACCAACGACGCGTTCCTGGCCGTTG
C T T C G T C C C T T T

751
ATGACGCGGTTGTGCGAGCCGGCGCACACTCGAAATCACTTGCTAATTCC
T T T T C G T C C

FIGURE 4D

002020" 4042F560

801
CTAGGCGATGACATCCCGCTCGATACCGAACGTGGATATCATATCGTCAT
T T AT G C C G
851
CGCGAATCCGGAAGCCGCTCCACGCATTCCGACGACCGATGCGTCAGGAA
C C A T T A T T T
901
AATTCATCGCGACACCTATGGAATGGGGCTTCGCGTGGCGGTACGGTT
G T T G T T T A C
951
GAGTTCGCTGGGCTCACAGCCGCTCCTAACTGGAACGTGCGCATGTGCT
T T T G T C T

FIGURE 4E

002020" 4042F960

1001

CTATACGCACGCTCGAAACTTCTTCCAGCCCTCGCGCCTGCGAGTTCTG
C T T GT G T T C

1051

AAGAACGATATTCCAAATGGATGGGTTCCGGCCGAGCATCCCGGATTCTG
T C G T T A A C

1101

CTCCCCGTGATTGGCCGGGCAACCCGGACACCCGACGTAATCTATGCTTT
T A T T T T T A T C

1151

CGGCCACGGTCATCTCGGCATGACAGGGCGCGCGATGACCGCAACGCTCG
T C T T T A C

FIGURE 4F

002020"4042F960

1201

TCTCAGAGCTCCTCGCAGGCGAAAGACCTCAATCGACATTTCGCCCTTC
T T T G T C T A

1251

GCACCAACCGGTTTGGTATTGGCAAATCCAAGCAACGGGTCCGGCAAG
T C T G T T TC

1301

TTAAGTGGGAATTCAAGCTTG
C

FIGURE 4G

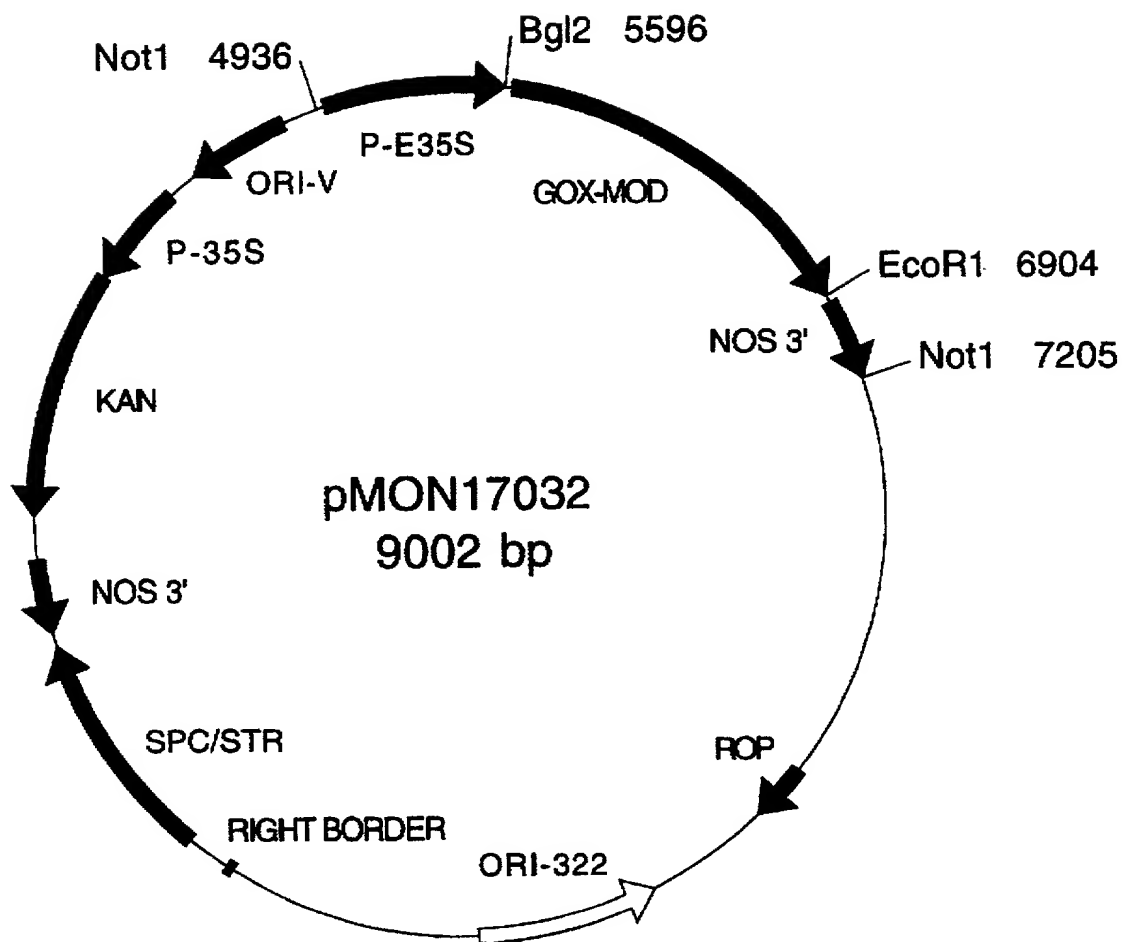


Figure 5

111	CCAGCCACCCGCAAGGCTAACACGACATTACTTCCATCACAAGCAACGGCGGA	164
	GGTCGGTGGCGGTTCCGATTGTTGCTGTAAATGAAGGTAGTGTTCGTTGCCGCCT	
	ProAlaThrArgLysAlaAsnAsnAspIleThrSerIleThrSerAsnGlyGly	
165	AGAGTTAACTGCATGCAGGTGTGGCCTCCGATTGGAAGAAGATTGAGACT	218
	TCTCAATTGACGTACGTCCACACCGGAGGCTAACCTTTCTTCTCAAACTCTGA	
	ArgValAsnCysMetGlnValTrpProProIleGlyLysLysLysPheGluThr	
219	CTCTCTTACCTTCCCTGACCTTACCGATTCCGGTGGTCGCGTCAACTGCATGCAG	272
	GAGAGAAATGGAAGGACTGGAATGGCTAAGGCCACCAGCGCAGTTGACGTACGTC	
	LeuSerTyrLeuProAspLeuThrAspSerGlyGlyArgValAsnCysMetGln	

FIGURE 6B

002020" 4042F950

N
 C
 O
 I
 GCCATGG
 273 -----+ 279
 CGGTACC
 AlaMet

FIGURE 6C

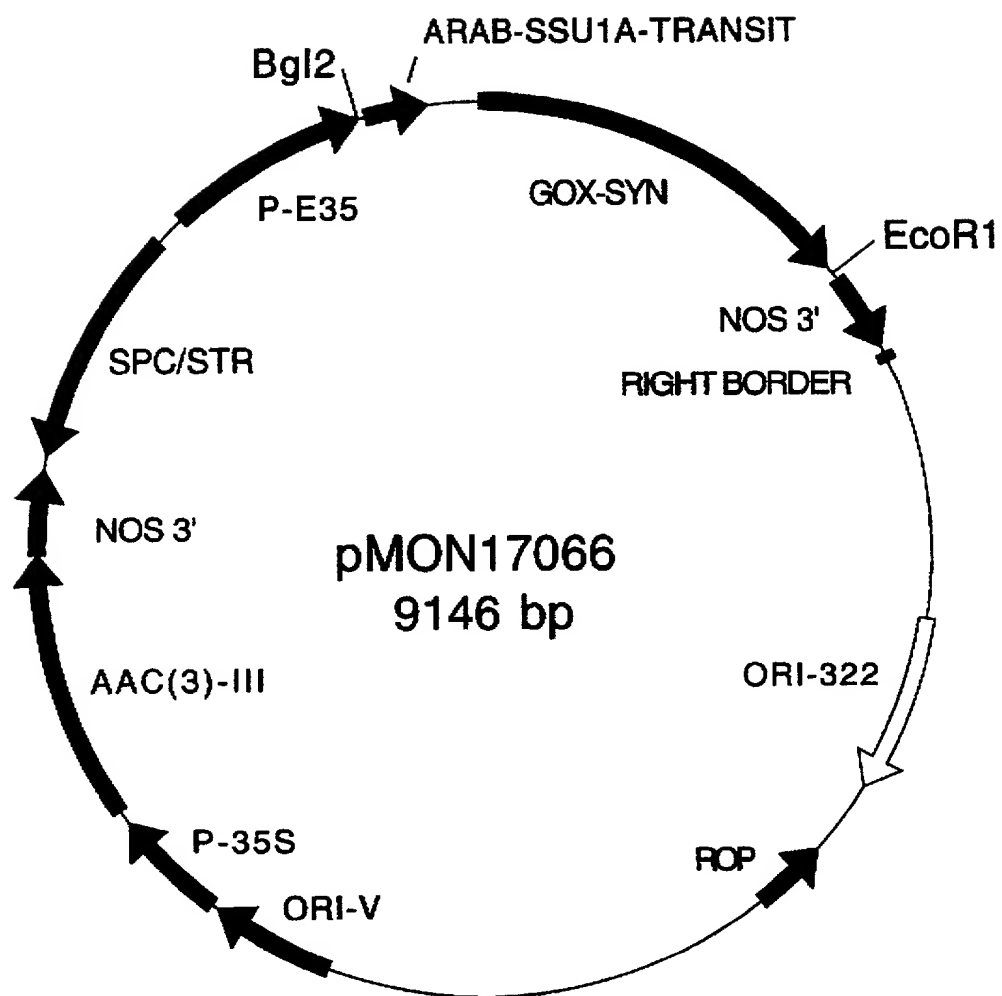


Figure 7

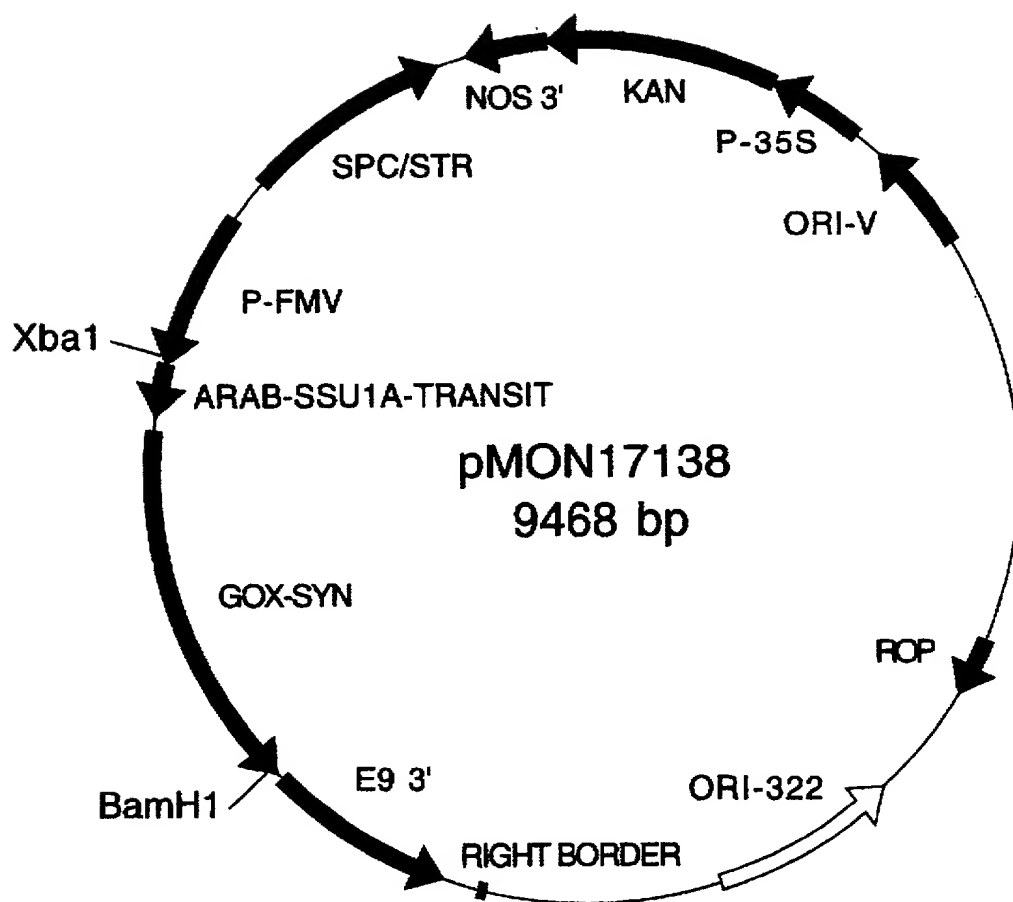


Figure 8

FIGURE 9A

S
p
h
I

CTTCGTCCTCTTAAGGTCA TGTCCTTCTGTTCCACGGCGTG CATGC

273

-----+-----+-----+-----+-----+-----+-----

GAAGCAGGAGAATTCCAGTACAGAAAGACAAAGGTGCCGCACGTACG

LeuArgProLeuLysValMetSerSerValSerThrAlaCysMet

FIGURE 9C

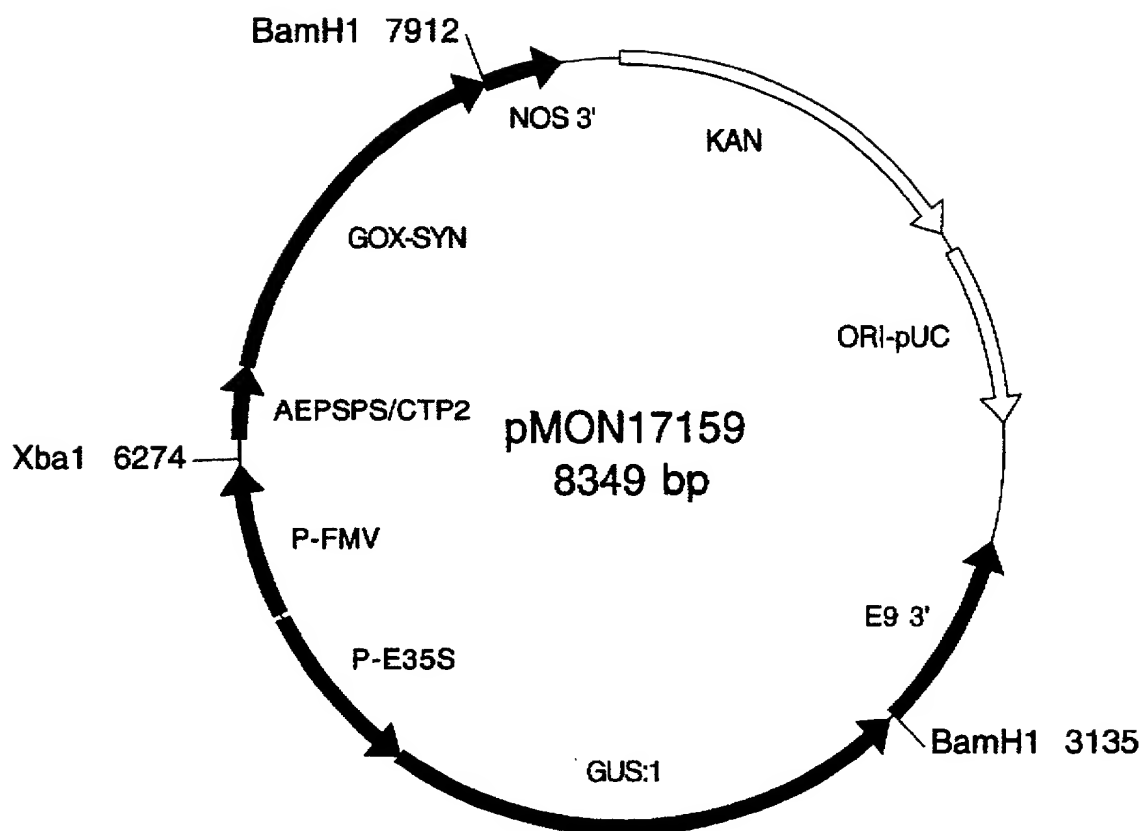


Figure 10

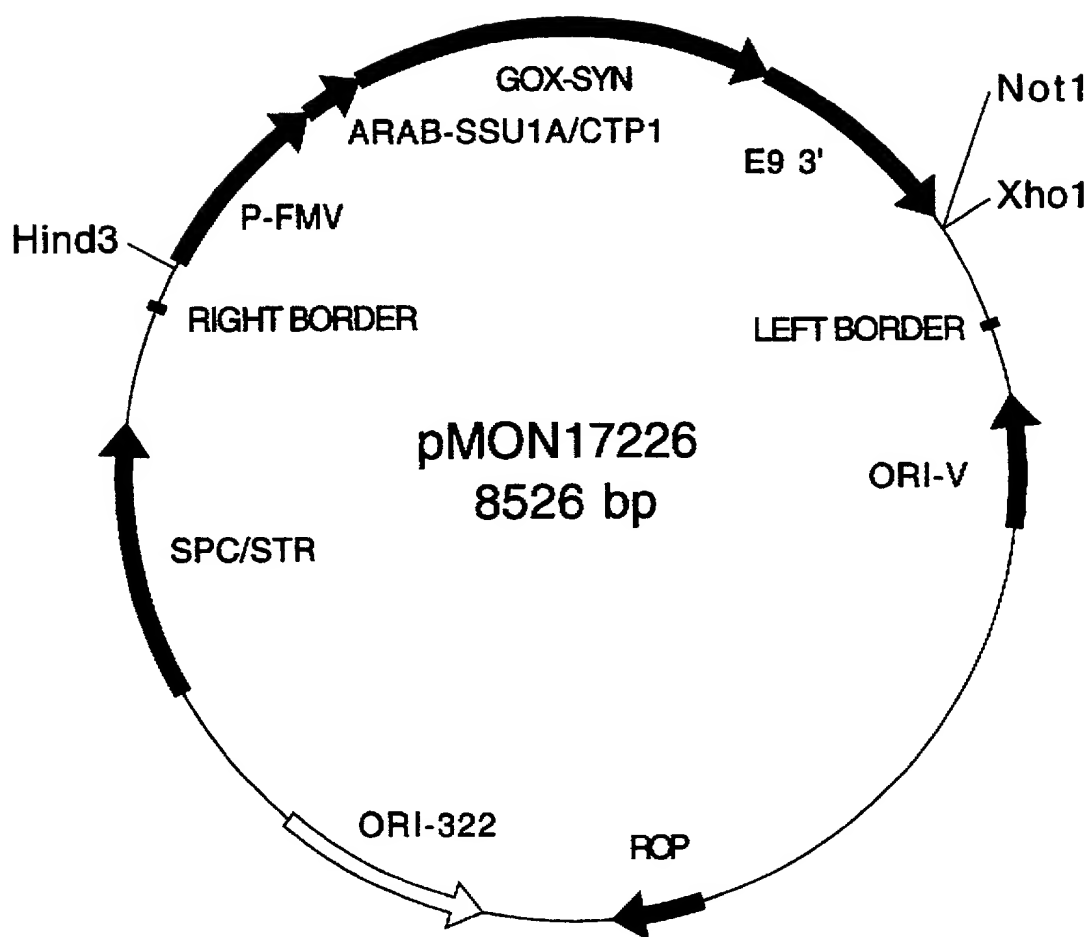


Figure 11

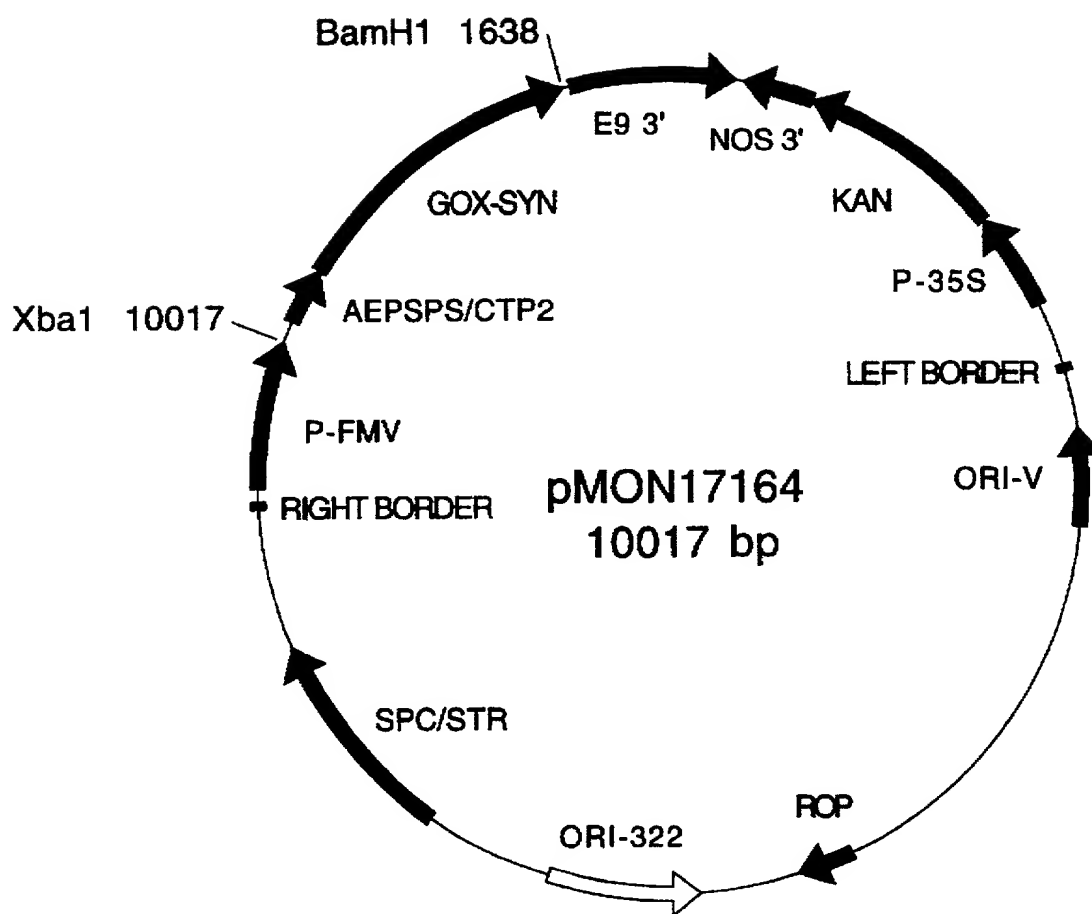


Figure 12

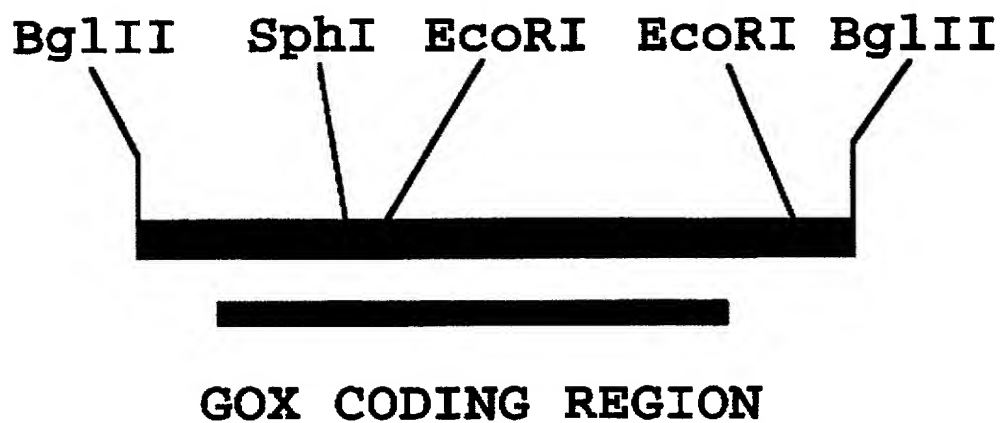


Figure 13



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REISSUE APPLICATION DECLARATION BY THE INVENTORS

Docket Number (Optional)
MONY:140

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is described and claimed in patent number 5,776,760, granted July 7, 1998, and for which a reissue patent is sought on the invention entitled "Glyphosate Tolerant Plants," the specification of which

☐ is attached hereto.

☒ was filed on July 7, 2000 as reissue application number 09/612,404
and was amended by preliminary amendment filed on _____.

I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56.

I verily believe the original patent to be wholly or partly inoperative or invalid, for the reasons described below. (Check all boxes that apply.)

☐ by reason of a defective specification or drawing.

☒ by reason of the patentee claiming more or less than he had the right to claim in the patent.

☐ by reason of other errors.

At least one error upon which reissue is based is described as follows:

The claims issued in U.S. Patent No. 5,776,760 did not claim or specifically claim certain DNA sequences, e.g., SEQ ID NOS:4, 6, 7, 8, and 17.

[Page 1 of 2]

Burden Hour Statement: This form is estimated to take 0.5 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Washington, DC 20231.

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(REISSUE APPLICATION DECLARATION BY THE INVENTOR, page 2)		Docket Number (Optional) MONY:140	
All errors corrected in this reissue application arose without any deceptive intention on the part of the applicant. As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.			
Name(s)		Registration Number	
Janelle D. Waack		36,300	
Melinda L. Patterson		33,062	
Correspondence Address: Direct all communications about the application to:			
<input type="checkbox"/> Customer Number	<div style="border: 1px solid black; width: 150px; height: 30px;"></div> Type Customer Number here	→	<div style="border: 1px solid black; padding: 5px; text-align: center;">Place Customer Number Bar Code Label here</div>
OR			
<input checked="" type="checkbox"/> Firm or Individual Name	Howrey Simon Arnold & White, LLP		
Address	750 Bering Drive		
Address			
City	Houston	State	TX
Country	USA		
Telephone	(713) 787-1400	Fax	(713) 787-1440
I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine and imprisonment, or both, under 18 U.S.C. 1001, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this declaration is directed.			
Full name of sole or first inventor (given name, family name): Gerard F. Barry			
Inventor's Signature		<i>Gerard F. Barry</i>	
Residence Address: 5555 Pershing Avenue, #2E St. Louis, Missouri 63112		Date: <i>Sept. 05, 2000</i>	
Post Office Address (if different from above):		Citizenship: Republic of Ireland	
Full name of second joint inventor (given name, family name): Ganesh M. Kishore			
Inventor's Signature		<i>Ganesh M. Kishore</i>	
Residence Address: 11966 Sackston Ridge Drive Creve Coeur, Missouri 63141		Date: <i>Sept 08, 2000</i>	
Post Office Address (if different from above):		Citizenship: United States	



**UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office**

ASSISTANT SECRETARY AND COMMISSIONER
OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

DATE: 10/08/91

TO:

DENNIS R. HOERNER, JR.
MONSANTO COMPANY-BB4F
700 CHESTERFIELD VILLAGE PARKWAY
ST. LOUIS, MO 63198

**UNITED STATES PATENT AND TRADEMARK OFFICE
NOTICE OF RECORDATION OF ASSIGNMENT DOCUMENT**

OCT 29 1991

THE ENCLOSED DOCUMENT HAS BEEN RECORDED BY THE ASSIGNMENT DIVISION OF THE U.S. PATENT AND TRADEMARK OFFICE. A COMPLETE MICROFILM COPY IS AVAILABLE AT THE U.S. PATENT AND TRADEMARK OFFICE ON THE REEL AND FRAME NUMBER REFERENCED BELOW.

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ASSIGNOR:

BARRY, GERARD F.

DOC DATE: 06/21/91

ASSIGNOR:

KISHORE, GANESH M.

DOC DATE: 06/21/91

RECORDATION DATE: 06/24/91 NUMBER OF PAGES 002 REEL/FRAME 5839/0986

DIGEST :ASSIGNMENT OF ASSIGNORS INTEREST

ASSIGNEE:

MONSANTO COMPANY, A CORP. OF DE

SERIAL NUMBER 7-717370 FILING DATE 06/24/91
PATENT PATENT ISSUE DATE 00/00/00

xc JS

PATENT
38-21(10533)A

NEW APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICATION OF

**GERARD FRANCIS BARRY and
GANESH MURTHY KISHORE and**

GROUP ART UNIT:

SERIAL NUMBER:

EXAMINER:

FILED: JUNE 24, 1991

JUNE 24, 1991

TITLE: GLYPHOSATE TOLERANT PLANTS

Commissioner of Patents and Trademarks
Washington, D. C. 20231
Sir:

Transmitted herewith for filing is the patent application of:

INVENTOR: GERARD FRANCIS BARRY and GANESH MURTHY KISHORE

TITLE: GLYPHOSATE TOLERANT PLANTS

Enclosed are:

- ☒ Abstract, Specification, Claims and attached Declaration.
- ☒ An Assignment of the invention to Monsanto Company, 700 Chesterfield Village Parkway, St. Louis, Missouri 63198 for recordation.

Also enclosed are:

- ☒ 14 sheets of 12 formal drawings.
- ☐ A certified copy of application.
- ☐ Prior Art Statement (37 CFR 1.97)

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U.S. DEPARTMENT OF JUSTICE

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717370

Return to:
Dennis R. Hoerner, Jr.
Monsanto Company
700 Chesterfield Village Parkway
St. Louis, Missouri 63198

Serial No.
Filed

ASSIGNMENT

WHEREAS, we, Gerard Francis Barry and Ganesh Murthy Kishore of 6350 Waterman Avenue, St. Louis, Missouri 631301, and 5354 Grantley Drive, Chesterfield, Missouri 63017, have invented certain improvements in GLYPHOSATE TOLERANT PLANTS identified as 38-21(10533)A and described in a patent application executed by us on the dates set after our signatures hereto; and

WHEREAS, Monsanto Company, of St. Louis, Missouri, a corporation of the State of Delaware, is desirous of acquiring the entire right, title and interest in and to said invention or inventions and any and all patents to be obtained therefor;

09612404-070700
NOW, THEREFORE, in consideration of five dollars (\$5.00), The receipt of which is hereby acknowledged, and other valuable consideration, we do hereby sell, assign and transfer unto said Monsanto Company, its successors and assigns, the entire right, title and interest in and to said invention or inventions, as described in the aforesaid application, in any form or embodiment thereof, and in and to the aforesaid application; and in and to any applications filed in any foreign country based thereon, including the right to file said foreign applications under the provisions of the International Convention; also any improvements on said invention or inventions now or hereafter made by us during the period of our employment; also the entire right, title and interest in and to any and all patents or reissues or divisional, continuation, continuation-in-part or substitute applications which may be filed upon said invention, inventions, or improvements in this or any foreign country; and we hereby authorize and request the issuing authority to issue any and all patents on said application or applications to said Monsanto Company, as assignee of the entire interest.

We further agree, without any payment by Monsanto Company other than expenses incurred by the undersigned to communicate to said Monsanto Company, its representatives or agents, any facts relating to any invention, inventions or improvements, including evidence for interference purposes or for other legal proceedings, whenever requested; testify in any interference or other legal proceedings, whenever requested; and execute and deliver, on request, all lawful papers required to make any of the foregoing provisions effective.

IN TESTIMONY WHEREOF, we have hereunto set our hands and seals on the dates set after our signatures.

(LS) Gerard Francis Barry, June 21 1991

REL 5839 NAME 987



UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office

ASSISTANT SECRETARY AND COMMISSIONER
OF PATENTS AND TRADEMARKS
Washington, D.C. 20231



SEP 27 1990

TO: DENNIS R. HOSKINS, JR.
MONSANTO COMPANY -BB4F
700 CHESTERFIELD VILLAGE PARKWAY
ST. LOUIS, MO 63198

UNITED STATES PATENT AND TRADEMARK OFFICE
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AND APPEARS IN THE OFFICE'S RECORDS AS SHOWN:

ASSIGNOR: 001 BARRY, GERARD F.
ASSIGNOR: 002 KISHORE, GANESH M.

DOC DATE: 06/25/90
DOC DATE: 06/25/90

RECORDATION DATE: 06/25/90 NUMBER OF PAGES 002 REEL/FRAME 5387/0078

DIGEST: ASSIGNMENT OF ASSIGNORS INTEREST

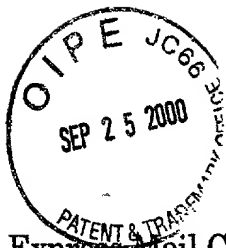
ASSIGNEE: 501 MONSANTO COMPANY, ST. LOUIS, MO, A CORP. OF DE

SERIAL NUMBER 7-543236 FILING DATE 06/25/90
PATENT NUMBER ISSUE DATE 00/00/00

002020-404240-061240

10/1/90

10/1/90



07 543236 *AD*

Express Mail Certificate #B50484568, mailed 6/25/90



PATENT
38-21(10518)A

NEW APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner of Patents and Trademarks
Washington, D. C. 20231
Sir:

Transmitted herewith for filing is the patent application of:

INVENTOR: Gerard Francis Barry and Ganish Murthy Kishore

TITLE: GLYPHOSATE TOLERANT PLANTS

Enclosed are:

- ☒ Abstract, Specification, Claims and attached Declaration.
- ☒ An Assignment of the invention to Monsanto Company, 700 Chesterfield Village Parkway, St. Louis, Missouri 63198 for recordation.

Also enclosed are:

- // ☒ Sheets of informal drawing.
- ☐ A certified copy of application.
- ☐ Prior Art Statement (37 CFR 1.97)

09612404-070700

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Return to:

Dennis R. Hoerner, Jr.

ASSIGNMENTS¹ No. _____

Filed _____

07 543236

Monsanto Company
700 Chesterfield Village Parkway
St. Louis, Missouri 63198

WHEREAS, We Gerard Francis Barry and Ganesh Murthy Kishore
_____, of 6350 Waterman, St.

Louis, Missouri 63130 and 15354 Grantley Drive, Chesterfield, Missouri 63017,
respectively

have invented certain improvements in GLYPHOSATE TOLERANT PLANTS

_____, identified as 38-21(10518)A

and described in a patent application executed by us on the date S set after our signature S hereto; and

WHEREAS, Monsanto Company, of St. Louis, Missouri, a corporation of the State of Delaware, is desirous of acquiring the entire right, title and interest in and to said invention or inventions and any and all patents to be obtained therefor;

NOW, THEREFORE, in consideration of Five Dollars (\$5.00), the receipt of which is hereby acknowledged, and other valuable consideration, we do hereby sell, assign and transfer unto said Monsanto Company, its successors and assigns, the entire right, title and interest in and to said invention or inventions, as described in the aforesaid application, in any form or embodiment thereof, and in and to the aforesaid application; and in and to any applications filed in any foreign country based thereon, including the right to file said foreign applications under the provisions of the International Convention; also any improvements on said invention or inventions now or hereafter made by us during the period of our employment; also the entire right, title and interest in and to any and all patents or reissues or extensions thereof to be obtained in this or any foreign country upon said invention, inventions, or improvements and any divisional, continuation, continuation-in-part or substitute applications which may be filed upon said invention, inventions, or improvements in this or any foreign country; and we hereby authorize and request the issuing authority to issue any and all patents on said application or applications to said Monsanto Company, as assignee of the entire interest.

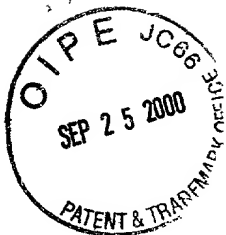
We further agree, without any payment by Monsanto Company other than expenses incurred by the undersigned, to communicate to said Monsanto Company, its representatives or agents, any facts relating to said invention, inventions or improvements, including evidence for interference purposes or for other legal proceedings, whenever requested; testify in any interference or other legal proceedings, whenever requested; and execute and deliver, on request, all lawful papers required to make any of the foregoing provisions effective.

IN TESTIMONY WHEREOF, we have hereunto set our hand S and seal S on the date S
set after our signature S

(L. S.) Gerard Francis Barry, June 25 1990

(L. S.) Ganesh murthy Kishore, June 25 1990

REEL 538



State of Delaware
Office of the Secretary of State

PAGE 1

I, EDWARD J. FREEL, SECRETARY OF STATE OF THE STATE OF DELAWARE, DO HEREBY CERTIFY THAT THE SAID "MONSANTO COMPANY", FILED A CERTIFICATE OF AMENDMENT, CHANGING ITS NAME TO "PHARMACIA CORPORATION", THE THIRTY-FIRST DAY OF MARCH, A.D. 2000, AT 2:15 O'CLOCK P.M.

AND I DO HEREBY FURTHER CERTIFY THAT THE AFORESAID CORPORATION IS DULY INCORPORATED UNDER THE LAWS OF THE STATE OF DELAWARE AND IS IN GOOD STANDING AND HAS A LEGAL CORPORATE EXISTENCE NOT HAVING BEEN CANCELLED OR DISSOLVED SO FAR AS THE RECORDS OF THIS OFFICE SHOW AND IS DULY AUTHORIZED TO TRANSACT BUSINESS.



Edward J. Freel

Edward J. Freel, Secretary of State

0341113 8320

001222724

AUTHENTICATION:

0413788

DATE:

05-02-00

002020-40427960



ASSISTANT SECRETARY AND COMMISSIONER
OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

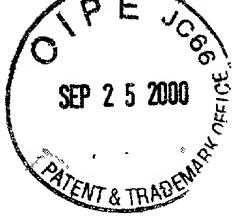
DENNIS R. HOERNER, JR.
MONSANTO COMPANY-BB4F
700 CHESTERFIELD VILLAGE PARKWAY
ST. LOUIS, MO 63198

OCT 29 1991

SERIAL NUMBER	7-717370	FILING DATE	06/24/91
PATENT PATENT		ISSUE DATE	00/00/00

55

4



17370

PATENT
38-21(10533)A

NEW APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICATION OF)

GERARD FRANCIS BARRY and)
GANESH MURTHY KISHORE and)

SERIAL NUMBER:)

FILED: JUNE 24, 1991)

TITLE: GLYPHOSATE TOLERANT)
PLANTS)

GROUP ART UNIT:

EXAMINER:

JUNE 24, 1991

Commissioner of Patents and Trademarks
Washington, D. C. 20231
Sir:

Transmitted herewith for filing is the patent application of:

INVENTOR: GERARD FRANCIS BARRY and GANESH MURTHY KISHORE

TITLE: GLYPHOSATE TOLERANT PLANTS

Enclosed are:

- ☒ Abstract, Specification, Claims and attached Declaration.
- ☒ An Assignment of the invention to Monsanto Company, 700 Chesterfield Village Parkway, St. Louis, Missouri 63198 for recordation.

Also enclosed are:

- ☒ 14 sheets of 12 formal drawings.
- ☐ A certified copy of application.
- ☐ Prior Art Statement (37 CFR 1.97)

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Return to:
Dennis R. Hoerner, Jr.
Monsanto Company
700 Chesterfield Village Parkway
St. Louis, Missouri 63198

Serial No.
Filed

ASSIGNMENT

WHEREAS, we, Gerard Francis Barry and Ganesh Murthy Kishore of 6350 Waterman Avenue, St. Louis, Missouri 631301, and 5354 Grantley Drive, Chesterfield, Missouri 63017, have invented certain improvements in GLYPHOSATE TOLERANT PLANTS identified as 38-21(10533)A and described in a patent application executed by us on the dates set after our signatures hereto; and

WHEREAS, Monsanto Company, of St. Louis, Missouri, a corporation of the State of Delaware, is desirous of acquiring the entire right, title and interest in and to said invention or inventions and any and all patents to be obtained therefor;

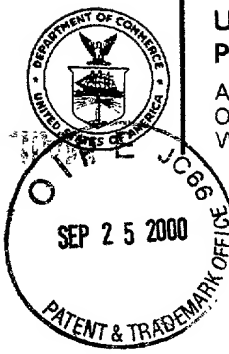
NOW, THEREFORE, in consideration of five dollars (\$5.00), The receipt of which is hereby acknowledged, and other valuable consideration, we do hereby sell, assign and transfer unto said Monsanto Company, its successors and assigns, the entire right, title and interest in and to said invention or inventions, as described in the aforesaid application, in any form or embodiment thereof, and in and to the aforesaid application; and in and to any applications filed in any foreign country based thereon, including the right to file said foreign applications under the provisions of the International Convention; also any improvements on said invention or inventions now or hereafter made by us during the period of our employment; also the entire right, title and interest in and to any and all patents or reissues or ex-divisional, continuation, continuation-in-part or substitute applications which may be filed upon said invention, inventions, or improvements in this or any foreign country; and we hereby authorize and request the issuing authority to issue any and all patents on said application or applications to said Monsanto Company, as assignee of the entire interest.

We further agree, without any payment by Monsanto Company other than expenses incurred by the undersigned to communicate to said Monsanto Company, its representatives or agents, any facts relating to any invention, inventions or improvements, including evidence for interference purposes or for other legal proceedings, whenever requested; testify in any interference or other legal proceedings, whenever requested; and execute and deliver, on request, all lawful papers required to make any of the foregoing provisions effective.

IN TESTIMONY WHEREOF, we have hereunto set our hands and seals on the dates set after our signatures.

(LS.) Gerard Francis Barry, June 24 1991

REF 5839 FRAME 987



UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
ASSISTANT SECRETARY AND COMMISSIONER
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Washington, D.C. 20231

SEP 27 1990

TO: DENNIS R. HOERNER, JR.
MONSANTO COMPANY -BB4F
700 CHESTERFIELD VILLAGE PARKWAY
ST. LOUIS, MO 63198

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ASSIGNOR: 001 BARRY, GERARD F.
ASSIGNOR: 002 KISHORE, GANESH M.

DOC DATE: 06/25/90
DOC DATE: 06/25/90

RECORDATION DATE: 06/25/90 NUMBER OF PAGES 002 REEL/FRAME 5387/0078

DIGEST: ASSIGNMENT OF ASSIGNORS INTEREST

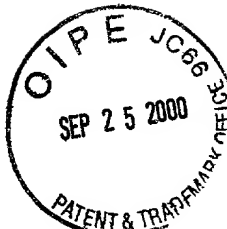
ASSIGNEE: 501 MONSANTO COMPANY, ST. LOUIS, MO, A CORP. OF DE

SERIAL NUMBER 7-543236 FILING DATE 06/25/90
PATENT NUMBER ISSUE DATE 00/00/00

0961244-0700

10/1/90

10/1/90



07 543236 AID

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PATENT
38-21(10518)A

APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner of Patents and Trademarks
Washington, D. C. 20231

Sir:

Transmitted herewith for filing is the patent application of:

INVENTOR: Gerard Francis Barry and Ganish Murthy Kishore

TITLE: GLYPHOSATE TOLERANT PLANTS

Enclosed are:

- ☒ Abstract, Specification, Claims and attached Declaration.
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- ☐ A certified copy of application.
- ☐ Prior Art Statement (37 CFR 1.97)
- ☐ Preliminary Amendment

The fee has been calculated as shown below.

Basic Filing Fee [37 CFR 1.16(a)]

\$370.00

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Dennis R. Hoerner, Jr.

ASSIGNMENT

Serial No. _____

Filed _____

07 543236

Monsanto Company
700 Chesterfield Village Parkway
St. Louis, Missouri 63198

WHEREAS, We, Gerard Francis Barry and Ganesh Murthy Kishore
_____, of 6350 Waterman, St.

Louis, Missouri 63130 and 15354 Grantley Drive, Chesterfield, Missouri 63017,
respectively

have invented certain improvements in GLYPHOSATE TOLERANT PLANTS

_____, identified as 38-21(10518)A

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IN TESTIMONY WHEREOF, we have hereunto set our hand S and seal S on the date S
set after our signature S.

(L. S.) Gerard Francis Barry, June 25 1990

REF 53